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THE BOTANICAL REVIEW

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# THE CHEMICAL MECHANISM OF PHOTOSYNTHESIS

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## INTRODUCTION

The significance of the process of photosynthesis is twofold. The importance of the production of organic matter from inorganic chemical compounds and the dependence of animals upon green plants for their food supply has been realised since the late eighteenth century. More recently the importance of photosynthesis as a mechanism in which chemical energy is produced from light energy with an efficiency unknown in any other photochemical reaction has been emphasized.

Plant physiologists at first considered photosynthesis as a process unique to green plants. Attempts to analyse it were made by studying the effect of a variety of external conditions upon the rate of the process. Such studies led F. F. Blackman (1905) to formulate a principle of "limiting factors" which was based upon the concept that photosynthesis involved both a photochemical and a thermochemical reaction. Many of the earlier investigations of photosynthesis were concerned with leaves of higher plants. In such a complex system as a leaf it is difficult to determine the light intensity or the concentration (or, more correctly, activity) of a

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reactant at the place of reaction as distinct from the more easily determined intensity and concentration at the morphological boundary. Only the former quantities are relevant in studies of kinetics, and hence it is desirable to investigate the kinetics of photosynthesis, using a system which approaches homogeneity. Later photosynthetic measurements were made, using a suspension of the unicellular alga, *Chlorella.* It can be shown that in a suspension of microscopic cells such as *Chlorella,* in contrast to the leaf of a higher plant, the concentration of carbon dioxide at the cell surface and possibly at the chloroplast surface is not appreciably different from the concentration in the external medium (Briggs, 1949). Investigations of the kinetics of photosynthesis resulted in the formulation of a photosynthetic mechanism consisting of at least three linked reactions. These were:

*a*) Formation of a complex between carbon dioxide and some intracellular substance, possibly chlorophyll.

*b*) Activation of this complex by light energy.

*c*) Breakdown of the activated complex with formation of reduced carbon compounds and the liberation of oxygen (Briggs, 1935).

Such a mechanism proved adequate for the mathematical formulation of photosynthetic phenomena. However, from the point of view of comparative biochemistry it appears probable that the actual mechanism may in its complete analysis consist of a large number of reactions similar in number to the reactions known to be part of aerobic respiration. Furthermore, the demonstration of carbon dioxide fixation by heterotrophic organisms has raised the question whether all of the partial reactions of photosynthesis are unique to green cells, or whether some might not be identical with reactions occurring in other metabolic processes. Van Niel (1949) has adduced further arguments for this point of view from studies comparing the photosynthetic activity of green plants and bacteria. He suggests that the primary photochemical reaction in the photosynthetic mechanism of these two groups of organisms might be the same but that the initial reactants and final products differ because the sequence of thermochemical reactions is different. The primary photochemical reaction common to all photosynthetic organisms consists, according to Van Niel, in the removal of hydrogen from water with the formation of a hydrogen donor which ultimately donates its hydrogen to carbon dioxide. As a conse-

quence of the removal of hydrogen from water there is formed a radical (more oxidised than water) which in the green plant breaks down with evolution of oxygen and formation of water. In the bacteria the radical does not break down with the evolution of oxygen but is reduced by an externally provided hydrogen donor, such as hydrogen sulphide or hydrogen. Gaffron (1944) has shown that, under certain conditions in the green plant, also, an uptake of gaseous hydrogen is associated with reduction of carbon dioxide. This was interpreted as a reduction of the postulated radical by hydrogen. Van Niel's suggestion was in some degree confirmed when it was shown, by the use of $O^{18}$ tracer, that the whole of the oxygen liberated in green plant photosynthesis probably came from the water molecules and that none came from the carbon dioxide (Ruben et al., 1941), a matter which will be discussed in detail in a later section. The reactions immediately concerned with the fixation and possibly also reduction of carbon dioxide are regarded as dark reactions, possibly analogous to the carbon dioxide fixation and reduction observed in non-pigmented organisms, such as chemosynthetic bacteria.

This viewpoint has become even more probable since it has been shown that suspensions of chloroplasts isolated from leaves, although unable to reduce carbon dioxide, can in light catalyse the reduction of a variety of hydrogen acceptors, in which reaction oxygen is evolved equivalent to the hydrogen acceptor reduced. In such photochemical reactions of chloroplasts, just as in photosynthesis, it has been shown that the oxygen evolved is produced entirely from water (Holt et al., 1948). Thus, isolated chloroplasts can be considered to contain that part of the mechanism of the green plant which is responsible for the primary photochemical reaction, although devoid of either or both of the catalytic systems concerned with the uptake and reduction of carbon dioxide.

If we regard photosynthesis as an oxidation-reduction reaction, the essential nature of which is the production by light of a hydrogen donor capable of reducing carbon dioxide, the minimum number of processes required are:

*a*) Absorption of light in a photochemical primary process.

*b*) Formation of a hydrogen donor with hydrogen ultimately derived from water.

*c*) Reduction of carbon dioxide by the hydrogen donor, directly or indirectly.

Chemically processes *a* and *b* may be represented in one reaction, e.g.,

*a* and *b*. $$A + [H_2O] \xrightarrow[\text{chlorophyll}]{h\nu} AH_2 + [O]$$

*c*. $$2\,AH_2 + [CO_2] \rightarrow [H_2CO] + 2\,A + H_2O$$

[] indicates that the molecules or atoms may exist as part of a complex. This review will consider each of these aspects of the photosynthetic mechanism. The photochemical primary reaction will be discussed with reference to observations upon the changes in fluorescence accompanying changes in photosynthesis; also with reference to the number of quanta and hence the number of photochemical primary reactions required for the evolution of one molecule of oxygen. The nature of the hydrogen donor ($AH_2$) concerned in photosynthesis will be discussed with reference to the effect of light upon the rate of reduction of various hydrogen acceptors by whole cells or by chloroplasts. Finally recent investigations which have attempted by the use of radioactive carbon dioxide to follow the "path of carbon" in photosynthesis will be considered.

Recent work in photosynthesis has been greatly stimulated by the interpretations of comparative biochemistry. In particular, work with chloroplasts and with radioactive carbon has increased our knowledge of many of the partial reactions which are part of, or are associated with, the photosynthetic mechanism. The integration of the partial reactions into a reaction mechanism of known topochemical features, such as was at one time the hope of the student of kinetics and constitutes the aim of the plant physiologist, still remains a problem for the future.

## THE PRIMARY PHOTOCHEMICAL REACTION

With the exception of the photosynthetic bacteria and the blue green algae, all photosynthesising cells contain chloroplasts, in which the chlorophyll and the carotenoid pigments are located—possibly highly concentrated in optically denser portions or "grana". Chlorophyll a is common to all photosynthesising cells of green plants, but, in addition, diverse pigments occur in different proportions in chloroplasts of different groups of organisms. The role of chlorophyll, apart from absorption of energy, and of the

other pigments is still doubtful; even for chlorophyll it has not been established whether the pigment acts only as a supplier of energy or also as a chemical catalyst. Recent investigations of the fluorescence of the pigments in the plant have added further to our knowledge both of the primary photochemical reaction and of the role of the pigments other than chlorophyll.

FLUORESCENCE STUDIES. Our present knowledge of the photochemistry of reactions involving complex molecules is still very limited, even when the molecules are free in solution (Livingston, 1949). Even if our knowledge were more extensive it would still be difficult to know how far the photochemistry of chlorophyll in solution could be applied to chlorophyll in such a complex structure as the chloroplast. Subsequent to extraction from the cell both the absorption and the fluorescence bands of chlorophyll shift towards shorter wavelengths compared to the position of the bands when the pigment is in the living cell. Furthermore the fluorescence of chlorophyll is from ten to 100 times weaker in the cell than when dissolved in organic solvents. These differences have been attributed to the fact that chlorophyll is not present in the chloroplast free in solution but is combined in a protein lipoid matrix whose structure is by no means certain. It is therefore clear that we are not yet in a position to analyse the photochemistry of chlorophyll in the chloroplast in precise physico-chemical terms.

Subsequent to absorption of light by a molecule, such as that of chlorophyll, the energy acquired changes the electronic state to that of an " excited " molecule. The " excited " or " activated " molecule has five possible fates, namely:

*a*) Emission of fluorescent light of wavelength longer than that of the incident light.

*b*) Collision with a suitable molecule resulting in chemical reaction.

*c*) Collision resulting in the degradation of energy as heat.

*d*) Spontaneous dissociation into atoms or radicals.

*e*) Changing its electronic state spontaneously, i.e., without collision, from that of an " excited " molecule to that of a molecule in a highly vibrating metastable state.

Process *e* has a finite probability only in polyatomic molecules. Subsequent to its formation in process *e* the highly vibrating meta-

stable molecule, which has a longer life than the primary excited molecule, becomes a normal non-excited molecule as a result of collision, in which energy is either dissipated as heat or used in a chemical reaction (probably different in type from the reaction resulting from process *b*). Franck (1951) has suggested that in the green cell process *e* alone results in photosynthesis, so we see that it is impossible to say what change in the intensity of fluorescence (process *a*) will occur as a result of a change in rate of photosynthesis unless it is known whether the rates of processes *b*, *c* and *d* have changed also. Only if the rate of processes *b*, *c* and *d* remain unchanged will a change in rate of photosynthesis be accompanied by an equivalent opposite change in fluorescence. If the small yield of fluorescence, observed to be of the order of 0.1%, is due to a large amount of energy being dissipated in other ways, then a small percentage change in the latter will result in a large percentage change in fluorescence. Franck (1949*a*) has clearly shown that, although many changes in rate of photosynthesis have been observed to be accompanied by changes of opposite sign in fluorescence, this is by no means always the case. Since we lack knowledge as to what factors determine the rate of each of these types of process, fluorescence studies alone can not provide an unequivocal explanation concerning any change in rate of photosynthesis.

A number of observations of the efficiency of fluorescence, i.e., the ratio of the intensity of fluorescent light to the intensity of incident light, have been reported both for leaves and algae. Fluorescence has been measured as the total intensity in an arbitrarily selected often very broad wavelength band. Rabinowitch (1950) has pointed out that one can only deduce values of the fluorescence efficiency from such measurements provided that during the period of observation no shift occurs in the position of the fluorescence bands. If it is assumed that no such shift occurs, the observations of fluorescence show that at low light intensities the fluorescence efficiency is constant. As the light is increased, an increase in fluorescence efficiency is observed, but ultimately, when the light intensity is increased still higher, the fluorescence efficiency approaches a new constant value, an increase of the order of 50%. The transition from the lower to the upper constant value occurs in some plants (e.g., *Hydrangea* leaves) at an intensity of the

same order as that at which photosynthesis is rapidly approaching its maximum rate (Franck et al., 1941; McAlister and Myers, 1940). In *Chlorella* the change in fluorescence efficiency occurs at a much higher intensity, measured with respect to the approach of photosynthesis to its maximum value. In any one organism the transition from the lower to the higher fluorescence efficiency occurs at lower light intensities, both in the presence of lower concentrations of carbon dioxide and in the presence of cyanide. (Shiau et al., 1947; but see also Wassink et al. (1943) whose work shows that the relationship between fluorescence and light intensity is more complex in diatoms than in *Chlorella* or *Hydrangea*). In the photosynthesis of purple bacteria which require a supply of hydrogen donor, such as thiosulphate, a similar change is observed with decrease in supply of hydrogen donor (Wassink, Katz and Dorrestein, 1941). Such effects might be due to a decreased supply of energy acceptors in the neighbourhood of the chlorophyll or the bacteriochlorophyll. Franck (1951) believes that this phenomenon can not be explained solely in terms of photosynthetic activity but that other photochemical reactions are concerned. An uptake of oxygen in the light by the green plant cell in the presence of high concentrations of carbon dioxide has been observed at very high light intensities. As the concentration of carbon dioxide is decreased so is the light intensity at which this photo-oxidative reaction becomes demonstrable (Franck and French, 1941). It has been suggested that this uptake of oxygen represents an oxidation of cellular substances by molecular oxygen photo-sensitised by chlorophyll. Franck postulates that in *Chlorella* and higher plants the presence of the products of this reaction in the neighborhood of chlorophyll increases the fluorescence efficiency of the chlorophyll—hence the observed relationship between the transition in fluorescence efficiency and the carbon dioxide concentration. A decrease in the concentration of oxygen would be expected to result in a decrease in rate of photo-oxidation, and it is found that the transition from the lower value of fluorescence efficiency to the higher occurs at a lower light intensity in air than it does in nitrogen (Shiau et al., 1947).

Fluorescence studies have also been made during the induction period of photosynthesis. The long induction period of photosynthesis following a long period of anaerobiosis is associated with

a high fluorescence yield. Franck (1949*a*) has postulated that as a result of fermentation in the dark during long periods of anaerobiosis, a substance similar in action to that resulting from photooxidation is produced. The presence of this substance is postulated to be responsible for the initial high fluorescence yield. It is further suggested that the substance is destroyed by oxygen so that the fluorescence will fall as the rate of photosynthesis rises. The changes in photosynthesis and fluorescence which occur subsequent to short periods of dark (" short induction "), consisting of a short sharp increase in fluorescence accompanied by a sharp temporary decrease in photosynthesis, have been explained by Franck as due to a temporary formation of a substance, in the presence of which the fluorescence efficiency of chlorophyll is increased and the photosynthetic activity is decreased.

As yet the postulate that there exist a number of compounds in the cell, produced as a result of dark reactions, which affect both the intensity of fluorescence and the rate of photosynthesis, constitutes an ad hoc explanation—the number of possible compounds being equal to the number of phenomena " explained ". As shown at the beginning of this section, it may prove impossible, with our present understanding of photochemistry, to obtain from studies of the relationship of rate of photosynthesis to intensity of fluorescence, fundamental knowledge of the photochemical primary reaction.

On the other hand, investigations of the fluorescence of chlorophyll when the cell is illuminated by light of wavelengths absorbed by some other pigment in addition to chlorophyll have provided considerable information as to whether energy is transferred from that pigment to chlorophyll. For such energy transfer to be possible the fluorescence bands of the absorbing molecule must overlap the absorption bands of the receiving molecule. Thus phycobilins, which absorb in the yellow green and fluoresce strongly in vivo in the orange and red, can transfer energy to chlorophyll, but since the absorption band of chlorophyll is in the red and its fluorescence band in the far red, the reverse is not possible. It has not yet been established whether light absorbed by phycoerythin in the red algae can excite chlorophyll fluorescence, although some preliminary observations bearing on this were reported (Van Norman et al., 1948). (We shall later discuss the

special relationship which appears to exist between phycobilins and chlorophyll in the red algae when we consider the results of measurements of quantum efficiency at different wavelengths in these algae). Transfer of energy from the carotenoids to chlorophyll in *Chlorella* has been shown by studying the effect of change in wavelength upon the yield of fluorescence. It was found that the yield of fluorescence declines very little as the wavelength of the exciting light changes from the region where it is almost completely absorbed by chlorophyll to a region where both carotenoids and chlorophyll absorb.

Even when 50% absorption is due to carotenoids, the fluorescence is reduced only 20%. It thus seems clear that energy transfer from excited carotenoids to chlorophyll occurs in the plant unless a change in wavelength of incident light is accompanied by a change in rate of those processes other than fluorescence which result in dissipation of energy. Similarly it has been shown (Wassink and Kersten, 1946) that in the diatoms energy can be transferred from fucoxanthol to chlorophyll. In a later section we shall discuss experiments in which the efficiency of photosynthesis in light of different wavelengths has been measured. Such experiments demonstrate that transfer of energy from the other pigments of the chloroplast to chlorophyll can occur with high efficiency.

Interpretation of such studies is, however, dependent upon the ability to apportion the light absorbed by the cell to the different pigments in the cell. This is a difficult task because the shift in the wavelength of maximum absorption and the sharpening of the absorption bands subsequent to extraction make it impossible to deduce with any great degree of certainty the absorption of the individual pigments in the cell from studies of extracts.

QUANTUM YIELD DETERMINATION IN RED LIGHT. According to Einstein, in the primary photochemical reaction each molecule of reactant transformed will require one quantum. Each mole quantum of red light of wavelength 6500 Å represents 43,000 cals., with corresponding larger amounts of energy for light of shorter wavelengths. Since for the reduction of each gram molecule of carbon dioxide reduced to the carbohydrate level $(CH_2O)_n$, 119,000 cals. free energy are required (in the standard state), at least three primary photochemical reactions must be involved in

the steady state of photosynthesis. Moreover, energy in excess of the difference in free energy will probably be required for "activation", and hence it is unlikely that less than four primary photochemical reactions occur. Since a minimum of four hydrogen atoms have to be transferred for the reduction of each carbon dioxide molecule, the process may occur in four equal steps, involving approximately the same free energy change and each requiring one or two quanta. Alternatively, the steps may be unequal, consisting, for example, of two reactions, each requiring two quanta together with two reactions, each requiring only one quantum. A mechanism of the latter type would require an overall quantum requirement of six. Mechanisms can thus be devised to accommodate any number of quanta greater than a minimum of four. Determinations of quantum efficiency are of use in limiting the possible types of mechanism of photosynthesis.

In the determination of quantum efficiency, which involves determination of the rate of photosynthesis at low light intensities, the problem as to whether the rate of respiration is affected by light intensity is particularly relevant. When respiration represents a small fraction of the total gaseous exchange in the light, an error in the value adopted for the rate of respiration will result in only a small error in the calculated value of real assimilation. When the apparent assimilation is near to zero, as is often the case in efficiency determinations, an error in the value adopted for the rate of respiration in the light will result in a large error in the calculated efficiency.

A change in the rate of respiration as a consequence of illumination may be due either to a change in the concentration of reactants in respiration, or to an effect of light upon the respiratory mechanism. In higher plants it has been demonstrated that after a period of illumination the respiration of leaves is increased and that this increase may persist for many hours (Audus, 1947). The increase cannot be ascribed simply to an increase in assimilates available as substrate. A similar effect has been observed with *Chlorella* (McAlister and Myers, 1940; Emerson and Lewis, 1939), in which case the magnitude and duration of the increase have been shown to be related to the period and intensity of illumination. It is also possible that there is an effect of light upon respiration during illumination which does not persist in the sub-

sequent dark period. Warburg et al. (Warburg and Burk, 1950*a*) have recently reported an attempt to determine the rate of respiration during illumination by comparing the gaseous exchange in light with that in the dark of *Chlorella* suspended in culture medium when the gas phase is in contact with alkali. When the system was well shaken the pressure changes in the light and dark were equal. Unfortunately these observations were confined to the special conditions, chosen by Warburg for another purpose, in which only one-twentieth of the total volume of the cell suspension was illuminated during the light. It appears extremely unlikely that the presence of sodium hydroxide in contact with the gas phase could have removed all carbon dioxide from the cells. Thus in the light almost certainly some photosynthesis occurred. If such is the case the experiments need careful analysis, since it appears that the experimental result could have been obtained only if the assimilatory quotient was far from unity or if some process other than photosynthesis and respiration took place in the light and consumed oxygen without uptake of carbon dioxide. Further experiments of this type, using a wide range of conditions and preferably analysing the gas phase for both oxygen and carbon dioxide, should be made. Experiments with mutant strains of *Chlorella,* which are unable to photosynthesise and whose respiration in the light can be compared directly with the dark rate, have been reported recently (Davis, 1950). It was found that light had no effect on the rate of oxygen uptake of these mutants. However, it is questionable whether these experiments are relevant to the discussion of the effect of light upon "endogenous" respiration, since the mutants were grown upon a medium containing glucose and have a considerably higher rate of respiration than that of *Chlorella* grown on mineral medium. Thus it is unlikely that the respiration of the mutant type and the endogenous respiration of the wild type represent the same process. It is also possible that photosynthesis and respiration in the light may have a common photochemical reaction which is "blocked" in the mutant. Only mutants blocked subsequent to this reaction can help to elucidate the problem of the effect of light upon respiration. Another recent attempt to determine whether respiration is the same in the light as in the dark has involved the use of two different isotopes of oxygen (Brown, 1950; quoted by Gaffron and Fager, 1951).

Since the oxygen evolved in photosynthesis originates from the water, whilst the oxygen consumed in respiration is largely taken from the gas phase, by using water containing $O^{18}$ and a gas phase containing $O^{16}$ it was possible to distinguish the oxygen exchange due to photosynthesis from that due to respiration. These experiments suggested that the rate of oxygen uptake due to respiration was the same in the dark as in the light in *Chlorella,* but that in other plants it might be appreciably different.

In order to minimize error due to changes in the rate of respiration, most of the determinations of quantum efficiency have been made using short alternating periods of light and dark. This may involve departure from the conditions of steady state photosynthesis. It will also introduce error if the time of response of the measuring system to light and to dark is of the same order as the periods of alteration.

The thermodynamic efficiency of photosynthesis will be a function of the initial reactants and final products. Franck (1949*b*) has pointed out that under certain conditions the process occurring in the light may not be the reduction of carbon dioxide but the reduction of some respiratory intermediate. As a specific example, Franck suggested that under normal conditions the processes of respiration and of photosynthesis are spatially separated in the cell. Under special conditions he considers it possible that the chloroplasts may become permeable to some intermediates of respiration. If respiratory intermediates are utilised in photosynthesis it might be expected that the observed quantum efficiency would depend on the relative magnitudes of photosynthesis and of the respiration; a considerable effect of light upon rate of respiration would also be expected. Kok (1948) had reported such an effect in *Chlorella,* i.e., he found a sharp break in the relationship between rate of photosynthesis and light intensity near the compensation point. Above the compensation point, when the rate of photosynthesis exceeded the rate of respiration, Kok found the efficiency of *Chlorella* suspended in alkaline solution to be 9 quanta/molecule oxygen and a little less than 9 quanta/molecule $O_2$ in acid solution. (These are not the published values which were in error due to a mistake in photometry, as noted by Rieke (see Franck 1949*b*)). Below the compensation point, when the rate of respiration is greater than the rate of photosynthesis, an

efficiency twice as great as this was observed. It is not clear whether in these experiments there was a determination of respiration appropriate to each measurement of photosynthesis. This is essential if respiration changes either with time or as a result of light exposures of different intensities. Furthermore there is as yet no evidence to indicate whether the results obtained by Kok, using relatively thin suspensions of *Chlorella,* can also be obtained using dense suspensions. Emerson and Lewis (unpublished data), using dense suspensions of *Chlorella* which absorbed almost all the incident light, found no evidence that the quantum efficiency changed when the light intensity was increased from a value below the compensation point to one above. Van der Veen (1949) also claimed to have shown with young wheat leaves an effect similar to that described by Kok in *Chlorella,* but further work has not confirmed the earlier observations. Additional investigations of the "Kok" effect are required before its significance can be evaluated.

The importance of determining whether light affects respiration and to what extent is well appreciated, but unfortunately there is no agreement between different investigators as to the method of measurement which will permit this effect to be allowed for in the determination of quantum efficiency. We shall consider the most recent investigations of the quantum efficiency of photosynthesis in red light and discuss possible causes as to why different investigators observe different values.

Some investigations of the quantum efficiency of photosynthesis have been made using leaves, but in this case it is difficult to estimate the light absorbed by the photosynthesising cells alone (Briggs, 1929; Gabrielson, 1940). It was estimated that 12 quanta or more were absorbed per molecule of oxygen produced.

If instead of leaves a suspension of algae is used, not only is it possible to stir the suspension so that on the average each cell is equally illuminated but also the presence of cells which absorb light but do not photosynthesise is avoided. Measurements with algal suspensions are conveniently made, using the manometric method. If the cells are suspended in alkaline solution the pressure change observed represents only the gaseous oxygen exchange. If the cells are suspended in acid solution, both the exchange of carbon dioxide and of oxygen contribute to the observed

pressure change, and in order to measure either of these quantities it is necessary either to assume a value for the assimilatory quotient or to use a "two-vessel" method. The latter technique is difficult, since it depends upon maintaining two vessels which differ in dimensions in an exactly similar state.

Warburg and Negelein (1922) first used a suspension of *Chlorella* cells for efficiency determinations and avoided the problem of determining the amount of light absorbed by using a suspension of cells so dense that absorption approached completeness. The cells were suspended in acid culture medium equilibrated with 5% carbon dioxide in air. The rate of pressure change was determined manometrically and it was assumed on the basis of measurements made under different conditions that the assimilatory quotient ($CO_2/O_2$) was near – 1.0. The cells were alternately illuminated and darkened for short periods (10–15 minutes), and the dark rate corresponding to the light period was taken as the mean of that observed in the preceding and following dark periods. Their results showed that as the light intensity was decreased, the number of quanta required to liberate one mole of oxygen approached the minimum number thermodynamically probable, namely, four.

Other workers later chose to use thin suspensions of algae. Using an integrating sphere to measure the fraction of incident light absorbed by the suspension and determining photosynthesis manometrically, Rieke (1949) found a consistent value of eight to ten quanta/mole oxygen with *Chlorella* cells suspended in alkaline solution. Other methods of measuring photosynthesis were used by different workers. Arnold (1949) measured photosynthesis by determining the thermal effect of the light absorbed in the presence and absence of photosynthesis. Eight to ten quanta/molecule of oxygen were required both in acid and alkaline solution. (Compare also the work of Tonnelat (1946)). Oxygen evolution in photosynthesis has also been measured, using the dropping mercury electrode (Moore and Duggar, 1949). Essentially the same value of about eight to ten, much lower than that observed by Warburg and Negelein, was found. Observations with algae other than *Chlorella* gave similar results.

There was therefore disagreement between the efficiencies observed with thin suspensions and the observations of Warburg and Negelein using thick suspensions in acid medium. Later work by

Warburg (1948) reaffirmed his earlier observations in acid solution but he too found in alkaline solution values of the order of ten. He attributed the difference in the two values to a deleterious effect of alkaline solution upon the photosynthetic mechanism of *Chlorella*. However, in the meanwhile Emerson and Lewis (1939) had repeated the work of Warburg and Negelein, using the same technique. They found that Warburg had incorrectly assumed that the assimilatory quotient of cells suspended in acid solution was – 1.0 during short periods of illumination. During the first minutes of illumination they found a considerable evolution of carbon dioxide (" burst "). In alkaline solution where the carbon dioxide is almost completely in solution and the pressure change is due to oxygen alone, no error of this sort arises. Emerson and Lewis found that the quantum requirement of cells suspended in alkaline solution was between eight and ten quanta/molecule oxygen. This value was in agreement with that found by all other workers and was later also confirmed by Warburg (Warburg and Burk, 1950*a*). Emerson and Lewis found further that if allowance were made for the carbon dioxide exchange in acid solution, the photosynthetic efficiency based upon evolution of oxygen was the same in acid as in alkaline solution. Their work suggested that the high efficiency claimed to have been found by Warburg was incorrect because part of the pressure change observed by Warburg and Negelein was incorrectly attributed to an exchange of oxygen instead of being analysed into one component due to oxygen and another due to carbon dioxide (Emerson and Nishimura, 1949).

Subsequent to the findings of Emerson and Lewis, Warburg (1948) repeated his earlier work. He found that with cells cultured according to his specification (which, however, were not cultured in the same way as those used in his earlier experiments), the carbon dioxide " burst " (" Emerson " effect) is small except at rather high light intensities. Furthermore Warburg et al. (Warburg and Burk, 1950*a*; Warburg et al., 1950*b*) have recently made further observations of quantum efficiency, in which the two-vessel method was used, so that simultaneous measurements of both carbon dioxide and of oxygen were obtained. Again quantum requirements of four quanta per molecule of oxygen were observed. These determinations are of interest for a number of

different reasons. First, they were made with an intense beam of red light which illuminated only a portion of the vessel bottom, whereas quantum yields have hitherto been determined with low light intensities. With rapid shaking the cells in a dense suspension will regularly change from light to dark during a period of illumination; thus, on the average, each cell experiences a long dark period alternating with each light exposure. Warburg suggests this may allow the concentration of reactants in photosynthesis to retain their dark value, even during the illumination period. According to Warburg, if a small beam is used with a dense suspension in this manner, maximum efficiency can be observed over a range of intensities and it is not necessary to extrapolate to zero intensity. Secondly, a convenient method of measuring light intensity was introduced, in which the oxidation of thiourea in pyridine photosensitised by ethyl chlorophyllide was measured manometrically. This system had been previously calibrated against a bolometer (Warburg and Schocken, 1949), and further calibration data were also obtained later by Burk and Warburg (1951). Thirdly, a new principle of alternation between white light and white plus red light was introduced. It was found that during the first few periods of illumination, values of quantum efficiency averaged four but that the efficiency rapidly declined with time when the cells were alternated between light and dark. If, however, an auxiliary white light was used so that the cells were alternately exposed to white and then white plus red light, the effect of the red light remained constant for long periods of alternations and gave a continued value of four quanta/molecule $O_2$ evolved.

In considering the most recent experiments of Warburg et al., Emerson et al. (Nishimura, Whittingham and Emerson, 1951) have pointed out that the two-vessel method is such that a small consistent error in the observed pressure changes will result in a large consistent error in the calculated photosynthesis. It is therefore important that the vessels be at all times in a comparable state, a condition which will be fulfilled only if special precautions are observed. It has been shown in Emerson's laboratory that results similar to those reported by Warburg can be obtained with *Chlorella* grown under a variety of conditions provided the precise technique used by Warburg is followed, but that the same cells

yield different results either if different shaped manometric vessels are used or if the sequence of time intervals of light or dark are different from those used by Warburg et al. According to Emerson et al., these different results arise because the pressure changes accepted by Warburg as representing the rate of photosynthesis are determined also both by the magnitude of the " burst " and by the difference in diffusion path in the two manometric vessels. The result of the combination of these quantities will be different for different vessels and will be a function of the period of alternation; in general the vessels will not be in a comparable state.

The most recent experiments of Burk and Warburg (1950, 1951) have revealed new hitherto unsuspected facts which, if confirmed, may alter our interpretation of the earlier measurements of photosynthetic efficiency. They claim to have shown that with *Chlorella* the observed quantum efficiency increases as the period of alternation between light and dark is shortened, finally attaining a value of unity with the shortest period of alternation (one minute). Nevertheless it is claimed that the quotient for both the light and dark reactions remained near unity. With longer periods of alternation the efficiency decreased to a value of three to five quanta/molecule oxygen with periods of seven to ten minutes. Emerson's objection that with brief periods of alternation the effect of diffusion can not be ignored in manometric determinations applies with even greater force to this latest work using very brief periods. A full appreciation of the significance of these latest data can come only as the result of further work, using the techniques described by Burk and Warburg, and, even more important, of an independent investigation of the phenomenon, using methods other than manometry. It is possible that the results might provide a solution to the problem of photosynthetic efficiency if it were established that with brief periods of light and dark a partial process of photosynthesis, with a quantum efficiency of unity, predominates, whilst in a steady state the quantum efficiency of the overall reaction is of the order of one-eighth. Warburg and Burk interpret their data by postulating that in green plants there is a combustion process which is induced by light and liberates energy used chemosynthetically; in very brief periods of alternate light and dark this reaction is maintained in such a way that most of the energy required for carbon dioxide assimilation

is obtained therefrom and only a small fraction is obtained from light energy.

If it is found that, in a steady state, the minimum number of quanta required per molecule of oxygen evolved is eight, there must be eight or less (if back reactions occur in reactions prior to the formation of hydrogen donator) photochemical primary processes involved in the reduction of one molecule of carbon dioxide to carbohydrate. This is in contrast to aerobic respiration, in which the oxidation of carbohydrate to carbon dioxide occurs via a number of linked reactions greater than eight. Most of the 119,000 calories liberated in the overall reaction are involved in the stepwise transfer of hydrogen to oxygen through a series of hydrogen carriers (pyridine nucleotide, flavo-protein, cytochrome) in reactions, some of which involve only 10,000 cals. An efficient reversal of such a system would require a supply of energy in units of this order of magnitude, and it is difficult to understand how this can be achieved with quanta, each representing 43,000 calories. The argument is even more powerful if only four quanta/molecule oxygen are required in the steady state, for then one hydrogen atom is transferred for each quantum. Thus it has been suggested that in photosynthesis transfer of hydrogen must occur in a single reaction involving a large free energy change, i.e., there must exist an oxidation reduction pair whose potential is more negative than that of any pair yet known in any biological system.

Transfer of hydrogen from carbohydrate to oxygen in a single large step is not unknown. Johnson et al. (1945) have suggested that the reaction resulting in luminescence in *Cypridina* and in the luminescent bacteria consists in the transfer of two hydrogen atoms from a compound almost as oxidised as carbohydrate directly to oxygen in a reaction which liberates, in the form of light, quanta of some 60,000 cals./mole. Thus, energetically the hydrogen chain in photosynthesis might be compared to a reversal of the reaction in bioluminescence rather than to the reactions of stepwise respiration. An alternative mechanism for the transfer of hydrogen in photosynthesis will be discussed in a later section.

Since the quanta of red light are so large in energy content compared with the difference in energy between a high energy phosphate ester and free phosphate, it seems unlikely that energy

is converted into phosphate bonds during the primary photochemical reactions with high efficiency. A difference in light and dark of phosphate exchange and redistribution in both green cells and purple sulphur bacteria has been observed (Wassink et al., 1949, 1951; Emerson et al., 1944), but we shall see in a later section that this is more likely to be concerned with carbon dioxide uptake than with the fixation of light energy as chemical bond energy.

QUANTUM YIELD DETERMINATION IN LIGHT OF DIFFERENT WAVELENGTHS. The light absorbed by each pigment as a fraction of the total light absorbed by the cell is a function of the wavelength of the incident light. Comparison of the absorption spectra with the spectrum of photosynthetic activity can provide information as to whether light absorbed by substances other than chlorophyll is utilised in photosynthesis. The difficulty with such measurements, as we have previously discussed in considering the transfer of energy from other pigments to chlorophyll, is to determine as a function of wavelength the relative absorption of the pigments in vivo. In interpreting measurements of quantum efficiency as a function of wavelength it is generally assumed that the efficiency of chlorophyll is independent of wavelength and hence of quantum size. Whilst this is a convenient supposition it is uncertain whether this is true at least in the far red beyond 6,800 Å where even at wavelengths for which the absorption of chlorophyll is still considerable the photosynthetic efficiency of the absorbed light appears to be very small (Emerson and Lewis, 1943). Experiments at these wavelengths, near to the infra-red, need special care, since it is possible that by the use of too wide a waveband of incident light an increase in wavelength will be accompanied by a gradual increase in the proportion of scattered infra red, resulting in the observation of a gradual decline in activity with increase in wavelength, although the actual decline is sharp.

In the green alga, *Chlorella,* the photosynthetic activity resulting from absorption of a given number of light quanta declines in the region of the spectrum where carotenoids absorb. The decline is not so great as would be expected from the relative absorption of the pigments in extract, assuming that only light absorbed by chlorophyll is effective in photosynthesis (Emerson and Lewis, 1943). It may be concluded that part of the energy absorbed by

the carotenoids is utilised in photosynthesis. Whether it is that energy absorbed by carotenoids as a whole is less effective photosynthetically than that absorbed by chlorophyll or that some of the carotenoids in *Chlorella* are completely inactive is not yet known. Fucoxanthol can be distinguished from the other carotenoids, since it absorbs at longer wavelengths. It has been shown in the diatom *Nitzschia closterium* (Dutton and Manning, 1941) and in *Navicula minima* (Tanada, 1951) that the light absorbed by fucoxanthol is about as effective in photosynthesis as that absorbed by chlorophyll. Of the phycobilins, Emerson and Lewis (1942) showed that light absorbed by phycocyanin in *Chroococcus* (with which organism a greater certainty in apportioning the light energy is possible, since the properly corrected combined absorption of extracts closely approaches the absorption curve of the whole cells) is almost as effective in photosynthesis as light absorbed by chlorophyll. If light absorbed by phycocyanin is effective in photosynthesis only when the energy is transferred via chlorophyll, a quantum absorbed by phycocyanin must have a high probability of being transferred to chlorophyll. Recently (Arnold and Oppenheimer, 1950) it has been shown that such a high probability is not incompatible with the expected probability based on a resonance transfer. This method of transfer of energy involves neither direct collision of the chlorophyll with excited phycocyanin molecules nor absorption by chlorophyll of the fluorescent light of phycocyanin—both of which processes are likely to have low probabilities. The efficiency of light absorbed by phycoerythrin compared to that of light absorbed by chlorophyll has been investigated in some marine red algae (Haxo and Blinks, 1950), and it appears that light absorbed by the phycoerythrin in the red algae is more effective than that absorbed by the chlorophyll. Indeed it appears that light absorbed by chlorophyll is used with very poor efficiency in these algae, although these plants can be adapted to efficient chlorophyll photosynthesis if they are kept in red light for some days (Blinks and Yocum, 1950). A possible explanation of these observations is that the phycobilins and chlorophyll compete for a limited surface, only in combination with which are they able to transfer energy to the photosynthetic reactants. Some evidence has been obtained in this laboratory (Greenham, unpublished) that all the chlorophyll in *Chlorella* is

not equally efficient, which can be interpreted in terms of a surface (or enzyme) that exists to only a limited extent. Duysens (1951) has also suggested that chlorophyll may occur in cells in "two different modifications".

Thus there is good evidence that the light absorbed by some pigments other than chlorophyll is utilised in photosynthesis. Further understanding of the problem of energy transfer might be obtained if the relationship between absorption of the pigments in vivo and in extract were better understood. Similarly further study of the decline of chlorophyll activity in the far red might add to our understanding of the nature of chlorophyll excitation.

## REDUCTION OF HYDROGEN ACCEPTORS OTHER THAN CARBON DIOXIDE

Studies of fluorescence can provide information concerning the reactions associated with the photochemical primary reaction. The problem remains as to the nature of the dark reactions of photosynthesis and of the enzyme systems concerned in those processes.

Associated with the chloroplasts one would expect to find those enzymes concerned with reactions immediately linked to the primary photochemical reactions. Many enzymes have been found in the chloroplast, but as yet the relationship of these to photosynthesis has not been demonstrated. Catalase has been reported in spinach chloroplasts (Neish, 1939), polyphenol oxidase in beet (Arnon, 1949) and a number of other plants (Warburg and Lüttgens, 1946), carbonic anhydrase in many but not all plants investigated (Bradfield, 1947). Sisakyan and co-workers' recent publications report several dehydrogenases in kidney-bean leaf, amongst which are glycine, glutamic, aspartic and malic, and with less activity citric and succinic dehydrogenases (1949*a*); they also report phosphorylase activity (1948*a*) and invertase, amylase and protease (1948*b*). Most of these determinations were made with isolated plastids. During the greening of barley, wheat and corn seedlings they find a twofold increase in catalase activity which occurs both in the presence and absence of carbon dioxide. (Compare similar work with corn by Eyster (1950)). Cytochrome oxidase activity was reported by this group (1949*b*) and also by others (Ducet and Rosenberg, 1951; du Buy, 1950). Perhaps of considerable significance for photosynthesis is the finding of R.

Hill (1949) that there is present in green leaves a cytochrome (called cytochrome-f) in addition to and distinct from the cytochromes found common to both green and colorless leaves. Cytochrome-f could not be removed by aqueous extraction and differs further from cytochrome-c by being less soluble in salt solutions and having a higher oxidizing potential. The function of this cytochrome in the chloroplast has not yet been analysed.

When chloroplasts are separated from leaves by fragmentation, it has yet to be demonstrated that the enzymes found in the sedimented chloroplast preparations were originally present only in the chloroplast. Warburg and Lüttgens (1946) found in spinach (*Spinacea oleracea*) that polyphenol oxidase activity remained in the precipitate (chloroplast fragments) only if extraction was in an acid medium. Later, Arnon (1949) found in spinach beet (*Beta vulgaris*) that even with extraction at pH 7.3 all activity remained in the precipitate. Arnon was, however, able to demonstrate that if preparations of polyphenol oxidase obtained from potatoes were added during the isolation of the chloroplasts, the added activity remained in solution. This was considered as evidence that the polyphenol oxidase found in the precipitate after extraction was present in the chloroplasts before isolation, or at least in discrete particles precipitated with the chloroplasts, and that it was not simply absorbed on to the chloroplasts during isolation; the argument is, however, not conclusive, as it has not been shown that the potato preparation had the same physical properties as the chloroplast preparation. This might not be the case if, for example, the protein combined with the prosthetic group is different in the two cases.

The first indication that carbon dioxide is not the only compound which can be reduced by green plant cells in light was obtained by Warburg and Negelein (1920). *Chlorella* cells suspended in a mixture of 0.1 M nitrate together with 0.01 M nitric acid evolved oxygen upon illumination. More recently it was shown that in the presence of certain hydrogen acceptors, e.g., benzaldehyde or nitro-urea, without added carbon dioxide, *Chlorella* cells evolved oxygen upon illumination (Fan et al., 1943). In the experiments of Warburg and Negelein, however, there was a considerable dark reaction resulting in the evolution of carbon dioxide. Although this was less than the oxygen evolved in light,

two possible interpretations of the effect of the light are possible, namely, the effect may be due either to assimilation of carbon dioxide produced by oxidation in the dark, which process may be stimulated by light, or to a direct reduction of the hydrogen acceptor by water using light energy. Similarly, the two possibilities also exist with the benzaldehyde reaction.

Hill and Scarisbrick (1940) showed that preparations of chloroplasts isolated from leaves after addition of certain hydrogen acceptors evolved oxygen in the light in amounts which could be measured manometrically. At first, oxygen evolution occurred only after addition of leaf extract to the chloroplast preparations, but it has now been shown that the leaf extract can be replaced by a wide variety of hydrogen acceptors. Tracer studies have shown that chloroplast preparations are unable to reduce carbon dioxide in the light (Brown and Franck, 1948) in spite of earlier claims to the contrary (Boichenko, 1943, 1944; Boyle, 1948). It is now considered probable that the photochemical oxidation reduction system of the chloroplast, although unable to reduce carbon dioxide, is the same as the photochemical oxidation reduction system of the whole cell which is involved in photosynthesis. Hence chloroplast preparations offer the first opportunity to study some part of the photosynthetic mechanism dissociated from cellular organisation.

REACTIONS OF ISOLATED CHLOROPLASTS. The simplest chloroplast preparations are made by grinding leaves in water, filtering through muslin, slow centrifugation of the filtrate, and then finally high speed centrifugation. The precipitate consists largely of a mixture of whole and fragmented chloroplasts. More uniform material can be obtained by successive grinding of the fragments with glass (Warburg and Lüttgens, 1946); by use of ultrasonic vibrations (French et al., 1941); or by forcing the chloroplast suspension through a small opening under high pressure (Milner, 1950*b*).

The initial activity (per unit of chlorophyll or protein) is dependent upon the species of plant (Clendenning and Gorham, 1950), upon the pretreatment of the leaves prior to fragmentation (Kumm and French, 1945) and upon the method of preparation. Good activity is obtained only if separation is done at temperatures near 0° C. Even so, the chloroplast preparations soon lose their

activity at room temperature, and even at temperatures near zero the activity persists for only a few hours. With each successive further fragmentation the activity of the preparation decreases. Extraction in 15% methanol (instead of aqueous sucrose solution) has been reported to stabilise the preparations against loss of activity in storage (Milner, 1950*a*); also "snap freezing" in dry ice followed by storage at temperatures as low as −40°C (Clendenning and Gorham, 1950).

Warburg and Lüttgens (1945) found that repeated washing of chloroplast preparations resulted in a progressive decrease in activity but that the loss could be restored by addition of either cytoplasmic fluid or of potassium chloride, bromide, iodide or nitrate in decreasing order of effectiveness. Arnon and Whatley (1949*a*) confirmed these results and were unable to find any stabilizing agent other than chloride in the cytoplasmic fluid. Chloroplasts obtained from "chloride free" plants showed little activity unless potassium chloride was added. They attributed the effect of chloride to a stabilising effect upon the chloroplast system in the light, which in the absence of chloride, whether in presence of a hydrogen acceptor or not, rapidly deteriorates. Addition of chloride was found to prevent loss of activity during storage in the dark to only a limited extent. Hill and co-workers, in their most recent paper (Hill, Davenport and Whatley, 1951), find that the activity of a crude chloroplast suspension decreases with washing, and they attribute this effect to the removal of a soluble factor which acts as a natural hydrogen acceptor. In these experiments no chloride effect was observed. It is clear that the rapid deterioration of activity makes detailed quantitative work with isolated chloroplasts difficult.

The range of substances which can be used as hydrogen acceptors will be determined by the energy required for their reduction and by the presence or absence of suitable catalysts. The reducing power of a compound compared to gaseous hydrogen in the standard state may be expressed in terms of the oxidation-reduction potential which is a measure of the free energy change of the reduction of the oxidised form by hydrogen. The potential is a function of the concentration of both oxidant and reductant. Further, if either the oxidant or reductant dissociates into charged ions, the potential is dependent upon the pH. For biological sys-

tems the potentials generally considered are those of the standard state at pH 7.0. Overall changes in the oxidation reduction potential of suspensions of cells, as distinct from chloroplast suspensions, have been measured (Wassink, 1947), but interpretation of such a change in a complex system like the cell is not easy.

Hill originally measured the oxygen evolution of the reaction by observing spectrophotometrically the formation of oxyhaemoglobin from haemoglobin. He also measured oxygen evolution by a manometric method. The use of dyes as hydrogen acceptors has made possible a rapid colorimetric method (Holt and French, 1948*b*) for determining the rate of reaction. Direct observations of change in the hydrogen ion concentration (Holt and French, 1946) or of the oxidation reduction potential (Spikes et al., 1950) have also been used. Some additional photochemical reaction between the hydrogen acceptor and naturally occurring hydrogen donors (e.g., ascorbic acid) present in the chloroplast preparation may occur, and care must be taken to allow for this in any rate determinations (French et al., 1950).

Hydrogen acceptors so far used in chloroplast reactions all have oxidation-reduction potentials more positive than carbon dioxide ($CO_2$ aq./HCOH aq. $= -0.5$ volts) *. Hill, using chloroplast preparations from *Stellaria* and *Chenopodium*, found ferric oxalate a suitable hydrogen acceptor. To prevent re-oxidation of the ferrous oxalate produced by the oxygen evolved in the reaction, Hill added potassium ferricyanide. Other workers, using chloroplasts from spinach (Holt and French, 1946) and spinach beet (Arnon and Whatley, 1949*b*), found that ferric oxalate was unnecessary, since hydrogen donation occurred directly to ferricyanide. It has not been shown whether this is due to the presence of small amounts of oxalate naturally occurring in these latter plants. In Hill's experiments ferric oxalate was reduced to about 95% with 4 mm. Hg oxygen pressure at pH 7.9, thus suggesting a maximum chloroplast reducing potential of about $-0.03$ volt. Cytochrome-c, which has a potential of $+0.26$ volt, can also be reduced in the chloroplast reaction (Holt, unpub.; Mehler, 1951), although evidence is still lacking as to whether reduction of cytochrome is accompanied by oxygen evolution. *o*-Benzoquinone can also serve as an oxidant (or hydrogen acceptor) in the chloro-

* $O_2$ electrode at pH $7 = +0.81$ volts.

plast reaction, the oxidation reduction potential of which at pH 7 (an optimum rate of reduction was observed between pH 6 and 7) is approximately + 0.36 volt. A number of substituted quinones have been used as hydrogen acceptor, but within the series the rate of reduction by illuminated chloroplasts is correlated only with the oxidation reduction potential at high light intensities (Aronoff, 1946). When a quinone or ferricyanide is used as a hydrogen acceptor, wavelengths which result in photochemical reaction of the oxidant itself must be excluded from the incident light. Oxidation reduction dyes, such as phenol indophenol and 2,6 dichlor phenol indophenol (+ 0.22 volt at pH 7 (Holt and French, 1948*b*)), can also be used as hydrogen acceptors. With the same chloroplast suspension all the different hydrogen acceptors, in the presence of stabilising potassium chloride, give similar yields of oxygen sometimes apparently approaching the theoretical maximum for the amount of hydrogen acceptor transformed (Arnon and Whatley, 1949*b*). Another reaction of interest, as we shall see when discussing the mechanism of carbon dioxide fixation in photosynthesis, is that addition of triphosphopyridine nucleotide (TPN) (− 0.30 volt at pH 7) to an unwashed crude suspension of chloroplasts greatly increases the evolution of oxygen in the light, the increase representing, for small concentrations of TPN some 30 times the equivalent of TPN added (Tolmach, 1951). Washed chloroplasts showed no reaction with TPN.

Davenport and Hill (1951) have drawn attention to the fact that the potential of cytochrome-f (+ 0.36 volt) is approximately halfway between that of oxygen and that estimated for the chloroplast system. They have suggested that hydrogen transfer in photosynthesis might occur in a number of steps, instead of one step involving a large free energy change, the first step involving a transfer of hydrogen from water to cytochrome-f, the second, transfer from cytochrome-f to some part of the chloroplast system. Rabinowitch (1945) has pointed out that by a process of energy dismutation a reaction can result in the formation of a hydrogen donator with only half the number of hydrogen atoms at twice the reducing potential. Thus in photosynthesis by a series of dismutations hydrogen might be transferred, first from water to cytochrome-f, then half of that hydrogen transferred to a chloroplast

reactant (potential about −0.03 volt), and then again half of that transferred to TPN, for example. Davenport and Hill point out that each of these transfers would involve about the same free energy change, and that, provided energetic coupling were one hundred per cent, only the first transfer need be a photochemical reaction. Such a process of energy dismutation must occur during chemosynthesis, as pointed out by Rabinowitch, for in that case the energy of oxidation of several molecules is used in the reduction of one carbon dioxide molecule. Studies of the quantum efficiency of the reduction of different hydrogen acceptors would provide information which might help to decide whether hydrogen transfer in photosynthesis is stepwise. The only objection that can be raised against this suggestion is that the primary photochemical hydrogen transfer must be a polymolecular reaction, the probability of which is small.

Concerning the effect of various inhibitors upon the chloroplast reaction some generalisations can be made, although the results of different workers do not show quantitative agreement. In agreement with the view that cyanide inhibits photosynthesis in a way other than by affecting the light reaction, this inhibitor has been found to have no effect on the chloroplast reaction. Hydroxylamine, which on the other hand has been considered to inhibit the reaction responsible for oxygen evolution in photosynthesis, inhibits the chloroplast reaction at concentrations of about $10^{-4}$ M. Azide also inhibits the chloroplast reaction in concentrations of $10^{-3}$ M (Macdowall, 1949). Earlier both Hill and Aronoff had found that neither hydroxylamine nor azide inhibited the reaction, but the absence of inhibition in these cases has been ascribed in the latter case to the low activity of the preparation (not stabilised by chloride) and in the former case to possible combination of the inhibitor with methaemoglobin used in the rate determination. Phenyl urethane inhibits to about the same extent as it inhibits photosynthesis. Warburg and Lüttgens (1946) showed that *o*-phenanthroline—a strong metal-binding agent—in almost equivalent amounts to the amount of zinc present in the chloroplast caused complete inhibition of the chloroplast reaction. Inhibition due to *o*-phenanthroline could be reversed by addition of a number of heavy metals which in stoichiometric amounts are effective in the order Ni and Co > Zn > Cu and Fe (Arnon and

Whatley, 1949*b*). More evidence is therefore required before we can deduce, as Warburg has suggested, that zinc is part of an enzyme essential to the chloroplast reaction; it appears probable, however, that the chloroplast reaction involves a catalytic metal complex.

The mechanism contained by chloroplasts able to produce a hydrogen donor in light has been compared to a part of the photosynthetic mechanism. As is true in photosynthesis, the oxygen liberated by chloroplasts all comes from water and none comes from the hydrogen acceptor (Holt and French, 1948*a*). Further many compounds previously considered to inhibit the photochemical reaction of photosynthesis were also found to inhibit the production of a hydrogen donor by chloroplasts in the light. Measurements of the yield of the reaction, although showing considerable scatter due to variation between successive chloroplast preparations, indicate a quantum requirement approximately the same as that of photosynthesis in alkaline solution (French and Rabideau, 1945). These facts, although not conclusive, suggest that the hydrogen donor formation in the reaction of chloroplasts may be identical with the photochemical primary reaction of photosynthesis, the difference being that in the latter case the hydrogen donor ultimately reduces carbon dioxide to carbohydrate.

USE OF HYDROGEN ACCEPTORS OTHER THAN CARBON DIOXIDE BY WHOLE CELLS. Subsequent to the use of various hydrogen acceptors in the chloroplast reaction, the same hydrogen acceptors have been used with whole cells. A further condition for reaction is now necessary, namely, that the hydrogen acceptors be able to penetrate into the cell. Warburg and Lüttgens (1946) obtained evolution of oxygen from whole *Chlorella* cells when illuminated in the presence of *o*-benzoquinone, and Aronoff (1946) found the same result with *Scenedesmus* cells. Others (Clendenning and Ehrmantraut, 1950) have used a mixture of ferric oxalate and ferricyanide (ferricyanide alone was not effective). Although the "chloroplast reaction" may be demonstrated with whole *Chlorella* cells, using quinone or ferricyanide, treatment with these compounds causes the cells to lose their ability to photosynthesise in bicarbonate/carbonate solutions. Whether this toxic effect is due to an interaction between quinone and alkali has not been shown. In 30 to 40 minutes illumination, during which the rate of oxygen

evolution was constant, Clendenning and Ehrmantraut found yields of some 80–85% theoretical, assuming that the whole of the hydrogen acceptor originally present had been reduced. The yield was consistently higher with quinone than with ferric salts. The reaction was inhibited by hydroxylamine, fluoride and sodium iodoacetate; it was not inhibited by cyanide. The maximum rate of oxygen evolution in high light, with the optimal concentration of quinone, approached the maximum rate of photosynthesis, although the quinone reaction needed a higher illumination to approach "saturation". Using intermittent illumination, consisting of brief flashes of the type used by Emerson and Arnold (1932) to study the kinetics of the dark reaction of photosynthesis, it was shown that the quinone reaction and photosynthesis have a dark reaction rate in common, which may be determined by a common photochemical reaction involving the formation of a hydrogen donor.

## PATH OF CARBON IN PHOTOSYNTHESIS

Hitherto we have discussed the role of light energy with respect to the formation of a hydrogen donor capable of reducing carbon dioxide. We have not yet considered possible mechanisms of incorporation of carbon dioxide into a form capable of being reduced by the cell. Since the work of Willstätter it has been supposed that in photosynthesis carbon dioxide is not reduced as such but first forms a complex with some cellular substance, which complex is then reduced by some "activated" compound, produced as a result of photochemical reaction, yielding reduced carbon compounds and free acceptor. The previous section has discussed experiments which suggest an interpretation of "activation" as the formation of a hydrogen donor. Three types of phenomena provide evidence for the possible existence of a carbon dioxide complex. First, the relationship found to exist between carbon dioxide concentration and rate of photosynthesis at high light intensities is evidence of the formation of a carbon dioxide complex. Secondly, after cessation of strong illumination an uptake or "pick-up" of carbon dioxide continues for several seconds (McAlister, 1939; Aufdemgarten, 1939); this has been interpreted as representing the regeneration of a carbon dioxide complex from the free acceptor released in the final reactions of photosynthesis. A third phenomenon is that during the first minutes of illumination

subsequent to an aerobic dark period there is a liberation of carbon dioxide lasting several minutes ("Emerson" effect or "burst") and a corresponding slow uptake or "gulp" of carbon dioxide after the cessation of illumination (Emerson and Lewis, 1941). It has been suggested that the "burst" is due to a photochemical decomposition of part of a carbon dioxide complex formed in the dark; however, the nature of the complex and whether it is directly related to the form of carbon dioxide reduced during photosynthesis has not been demonstrated. Whereas the time of the "burst" and "gulp" is of the order of minutes, the "pick-up" lasts seconds. However, the quantity of carbon dioxide evolved in the "burst" and taken up in the "gulp" and also the quantity evolved in the "pick-up" are of the same order as the amount of chlorophyll.

Since isolated chloroplasts are unable to reduce carbon dioxide in the light, either carbon dioxide uptake must take place outside the chloroplasts or some mechanism has been destroyed during extraction of the chloroplasts. The first alternative was shown to be more probable as the result of work by Frenkel (1941) who studied the uptake of radioactive carbon in *Nitella*. All the radioactive carbon taken up in the dark was found to be located in the cytoplasm, whereas after uptake in the light four-fifths of the activity was in the chloroplasts. The simplest interpretation of Frenkel's results is that carbon dioxide uptake occurs in the cytoplasm, whereas reduction of carbon dioxide is associated with the chloroplast, although the possibility is not excluded that there is an acceptor in the chloroplast which is small in comparison in the dark to the total acceptors for carbon dioxide in the cytoplasm.

It is now clear that incorporation of carbon dioxide into cell substances in the dark is not confined to green plants but occurs also in bacteria and animals. Such incorporation of carbon dioxide with the formation of carboxyl groups is a process to be clearly distinguished from reduction of carbon dioxide. The former process involves an energy change of only a few thousand calories and might occur at the expense of energy produced in the oxidation of other organic matter.

Recent investigators have studied in detail the compounds into which radioactive carbon is incorporated in both plant and animal cells in the dark. Two different types of mechanisms have been demonstrated:

*a*) Reductive carboxylation, e.g., the addition of carbon dioxide to pyruvic acid in the presence of "malic" enzyme, reduced TPN and manganese to form *l*-malic acid. The "malic" enzyme first isolated from animal tissues has been shown to be present in a number of plant tissues (Conn, Vennesland and Kraemer, 1949). Illuminated grana from spinach leaves are known to reduce TPN at least to a limited extent, and it has been demonstrated that addition of "malic" enzyme and pyruvate to illuminated grana suspensions results in the fixation of carbon dioxide and the formation of *l*-malate (Vishniac and Ochoa, 1951; Tolmach, 1951; Arnon, 1951).

$$CH_3COCOOH + CO_2 + \text{reduced TPN} \rightleftharpoons COOHCH_2CHOHCOOH + TPN$$

pyruvic acid — *l*-malic acid

*b*) Utilization of a phosphate bond in such a reaction as that between formic acid (in equilibrium with carbon dioxide and water) and acetyl phosphate to give pyruvic acid and free phosphate (Utter et al., 1945).

The reversal of $\beta$ decarboxylation (i.e., the $\beta$-decarboxylation of oxaloacetic acid to give pyruvic acid in the reaction

$$COOHCH_2COCOOH \rightarrow CH_3COCOOH + CO_2)$$

which was at one time thought to have been demonstrated (Wood Werkman reaction) is now thought to be improbable (Ochoa, 1951), and an alternative explanation of the earlier results is given in terms of the "malic" enzyme. Ochoa has suggested possible combinations of reactions which would provide a mechanism for the formation of three and four carbon atom acids from carbon dioxide and suitable carbon precursors. It is possible that such reactions may be concerned in the fixation of carbon dioxide prior to its reduction in photosynthesis to the carbohydrate level.

If this is the case, another role of phosphate in photosynthesis, distinct from any role in the photochemical primary reaction mentioned earlier, is possible, namely, in the incorporation of carbon dioxide. Vogler et al. (1942) have demonstrated that phosphate is concerned in the uptake of carbon dioxide in the chemosynthetic bacteria (*Thiobacillus thiooxidans*), since they could show that carbon dioxide uptake was much greater after a period of oxidation of external hydrogen donor in the absence of carbon dioxide

during which organic phosphate had been formed from inorganic. If it should be found that in photosynthesis also, phosphate esters are utilised in the formation of a carbon dioxide complex, such esters could arise either from the oxidation of carbohydrate or directly with the help of light energy. We have already seen that if the latter is the case this process is likely to have a very low efficiency, since the maximum utilisable energy associated with the known phosphate bonds is much less than that of a quantum.

After it was realised that carbon was incorporated by green plant cells into a number of different compounds in the dark the problem arose as to whether carbon incorporation is of the same nature in photosynthesis as in the dark reactions. Van Niel (1949) has argued from analogy with the reactions of the photosynthetic bacteria that it is probable that the same enzymic mechanism of carbon dioxide incorporation occurs in the light and dark. Barker et al. (unpublished) showed that in the non-purple sulphur bacteria assimilation of labelled carbon dioxide in the presence of acetate was quantitatively the same in the light as in the dark, i.e., the same proportion of carbon was incorporated into cellular material from the carbon dioxide and from the acetate. Some criticism and comments have recently been made by Kamen (1950).

It is possible to interpret the results of experiments using tracers unequivocally only if the order of the rates of all the possible exchange reactions between the labelled atom and other compounds present in the system containing this atom are known. Before discussing the results with tracer carbon upon the "carbon path of photosynthesis" we shall discuss the less complex results using tracer oxygen to illustrate the problems of interpretation of tracer experiments. In order to determine the source of the oxygen evolved in photosynthesis *Chlorella pyrenoidosa* was allowed to assimilate in bicarbonate carbonate buffer solutions in which the oxygen either of the water or of the carbonate contained some $O^{18}$. It was found that the isotopic composition of the oxygen evolved in photosynthesis was always the same as that of the water (Ruben et al., 1941). It was also shown that the rate of equilibration of oxygen between carbon dioxide and water in the absence of cells was much less than the rate of photosynthesis so that the result could not be due to an initial equilibration during which the isotopic composition of the oxygen of both bicarbonate

and water approached that of the original water present (in large excess). Hence it was concluded that the oxygen evolved in photosynthesis is derived solely from water molecules. If such is the case one might expect that the isotopic content of the oxygen of the air should be almost the same as that of the oceans. A small difference might be expected due to preferential isotopic evaporation. Experimentally, however, it was found that the $O^{18}$ content of the air was greater than that of the ocean and by an amount that could not be explained in terms of the known exchange reactions. Consideration of the earlier experiments with *Chlorella* shows that the interpretation depends upon two assumptions (Kamen and Barker, 1945). First, that exchange of oxygen between carbon dioxide and water in the presence of *Chlorella* cells is not considerably faster than that measured in their absence. Experiments using carbon dioxide and water in isotopic equilibrium, in which state the carbon dioxide has a greater $O^{18}$ content than the water, gave the same result as the earlier experiments (Dole, 1944), indicating the absence of any rapid exchange. Secondly, although in solution there is no evidence for isotopic equilibration between oxygen and water, this process might be catalysed by *Chlorella*. If this were so the oxygen produced in photosynthesis would equilibrate with water before evolution. This possibility has not yet been experimentally tested. However, there has recently been demonstrated the possibility of a different explanation of the geochemical discrepancy. It has been shown that there is an isotopic random exchange between carbon dioxide and water under the influence of ultra-violet light (Roake and Dole, 1950). Such a reaction could proceed in the stratosphere, and one might expect that in the absence of vertical mixing, the air near the ocean surface would have an oxygen content approaching that of the ocean, whereas towards the stratosphere the isotopic content would be higher. Thus in the absence of any evidence demonstrating a catalytic effect of *Chlorella* upon the exchange of oxygen between oxygen and water it seems reasonable to suppose that the oxygen evolved in photosynthesis does originate solely from the water. It is seen from this example that the interpretation of such experiments in which tracers are used is not simple, and the importance of any possible exchange reactions must not be overlooked.

Criticism may also be made of the assumption common in recent work with radioactive carbon that the first substance in which activity appears must be the first intermediate in the process. Whilst this may be so it can be shown from simple kinetic arguments that it is not necessarily the case. Further, in much recent work, determinations have been made of the percentage distribution of tracer without investigation as to whether also the total amount of the substance labelled has changed. The determination of specific activities rather than percentage distribution is important for discussion of the mechanism responsible for the labelling sequence. Having pointed out some difficulties in the interpretation of isotope work, we shall review the experiments with $C^{14}$ so far reported, which, whilst limited in their scope, have stimulated new suggestions as to the path of carbon in photosynthesis.

In an attempt to investigate the effect of light energy upon the carbon compounds present in the plant, Ruben et al. (1940) added the short lived tracer $C^{11}$ to photosynthesising *Chlorella* cells. Whereas in the dark the tracer carbon was found largely in the carboxyl groups of a compound of high molecular weight, reported as about 1,000 but with the possibility of very considerable error, in the light most of the activity was no longer in the carboxyl groups. In the dark uptake was slow, taking up to two hours to attain a stationary value, at which time the amount incorporated was of the same order as the amount of chlorophyll present. Evidence that the compound formed in the light was related to photosynthesis was twofold. First, the rate of carbon dioxide uptake was calculated from radioactivity measurements and found equal to the rate of photosynthesis measured manometrically. This is evidence of the absence of exchange reactions provided there is no selective utilisation of any one isotope. Weigl and Calvin (1949) made simultaneous measurements of carbon dioxide concentration of radioactivity and of specific activity of the gas phase containing 2% $C^{14}O_2$ in an enclosed system containing barley seedlings. After some minutes of dark the barley was illuminated and the specific activity was observed to rise, indicating a possible preferential uptake in the light of $C^{12}O_2$ over $C^{14}O_2$. Evidence of a quite different type (Nier, 1950) suggests that preferential uptake of $C^{12}$ over $C^{13}$ may occur in the light and hence that it is also possible that $C^{12}$ is preferred over $C^{14}$. Secondly, after treatment with either ultra-violet light or after addition of high concentra-

tions of cyanide ($10^{-2}$ M), both the rate of labelled carbon uptake and of photosynthesis were inhibited almost completely, whereas the rate of respiration was unchanged. Recently Allen, Guest and Kamen (1947), using $C^{14}$, a long lived tracer, have shown that inhibition by cyanide does not always result in similar reduction of both carbon uptake and of photosynthesis but that it is possible to stimulate radioactive carbon uptake whilst inhibiting both photosynthesis and respiration.

Brown, Fager and Gaffron (1949) have also attempted to separate the carbon dioxide uptake related to photosynthesis from that due to participation in respiratory reactions together with other dark processes not part of photosynthesis. They showed considerable uptake of carbon dioxide in the dark under both aerobic and anaerobic conditions, but no net carbon dioxide was assimilated in these reactions; the uptake was simply the result of exchange. In the light, as previously found by Ruben, the uptake was much greater and approximately equal to the carbon dioxide assimilated. This, however, does not exclude the possibility of exchange with compounds formed in the light, and exchange might be particularly important during the initial rapid fixation. These workers found that after the first few seconds of illumination the tracer was fixed almost completely in a water-soluble fraction stable to dilute acid. After a longer period of illumination activity was also found in compounds insoluble in aqueous alcohol (e.g., proteins, polysaccharides), whilst the activity of the water-soluble fraction was found to decrease. Similar results have been obtained by Calvin et al. However, in the presence of excess cyanide, when photosynthesis is below the compensation point, the activity of the water-soluble fraction has been found to be independent of time of illumination (Kamen, 1949). At first the water-soluble compounds resulting from tagging in the light were considered by Gaffron et al. to be stable in the dark, which was not true for the products of dark tagging, but it was later realised that this difference might be due to the fact that the large amount of insoluble tagged compounds formed in the light may change in the dark into water-soluble compounds, whilst the original water-soluble compounds were respired.

Calvin et al. (Calvin, 1949) have shown that the carbon dioxide uptake in the dark, subsequent to a period of illumination of some 30 minutes in the absence of carbon dioxide and in the presence

of nitrogen, consisted of a large and rapid uptake, lasting in some experiments only a few seconds, followed by a slow continuous uptake lasting many hours. Without pre-illumination uptake in the dark was similar to the second slow uptake, the initial rapid uptake being absent. The rapid dark fixation subsequent to illumination may be compared to the " pick up " observed with non-radioactive carbon dioxide which we have discussed earlier. The " pick up " was attributed to the existence of free carbon dioxide acceptor persisting for some seconds after the cessation of photosynthesis. There is an alternative mechanism possible, namely, that there might be formed during illumination some long-lived reducing system. Experiments with oxidation-reduction dyes failed to produce evidence in support of this (Fager et al., 1950). Fager et al. also showed it to be unlikely that the uptake of tagged carbon was due to reversals of decarboxylations arising from the deficiency of carbon dioxide created during illumination, since, even when pre-illumination occurred in the presence of excess carbon dioxide, an appreciable extra amount of tracer, equivalent to ten seconds photosynthetic fixation, was fixed in a subsequent brief dark period. The ability to fix carbon dioxide rapidly diminished in the dark, having a half life of only a few seconds. Fager et al. concluded that the most likely explanation was in terms of the existence of some free carbon dioxide acceptor persisting for some seconds after the cessation of illumination.

A discrepancy exists between these observations with tracer carbon and earlier investigations of McAlister (1939) with non-radioactive carbon dioxide in which it was reported that no appreciable " pick up " occurred after a period of photosynthesis at a high rate. Further work upon the " pick up " is necessary.

During their studies of the fixation of $C^{14}$, Calvin et al. investigated with Chlorella and with barley the products of fixation both in the dark, in the dark immediately after 5 to 10 minutes illumination in helium gas and in the light. They showed that some compounds, e.g., alanine, serine, aspartic acid, malic acid, were labelled under all of these conditions. Other compounds showed marked differences as a result of these treatments. In *Chlorella* labelling during a period of 30 seconds photosynthesis followed by 150 seconds in the light in an atmosphere of helium produced little activity in either glutamic or isocitric acids, whereas labelling

during 30 seconds photosynthesis followed by 150 seconds in the dark in an atmosphere of helium showed considerable activity in both glutamic and isocitric acids. The activity in these acids was increased still further if the dark period was aerobic and not anaerobic. Benson and Calvin (1950*b*) have suggested that light must therefore not only initiate photosynthesis but also result in inhibition of the formation of tricarboxylic acids, such as glutamic and isocitric acids. However, in barley, even after long dark periods in the presence of tracer, little glutamic acid is found labelled so that this effect, described for *Chlorella,* is absent. It is clear that light may cause redistribution of $C^{14}$ in ways other than by the reactions of photosynthesis. Calvin et al. (1951) suggest that only compounds labelled in the light or in the dark immediately after illumination are definitely concerned in photosynthesis; those compounds which are also labelled in the dark (not after pre-illumination) may be concerned in either respiration or photosynthesis or both.

Extensive specific identification of compounds tagged during photosynthesis and in the dark, after pre-illumination in the absence of carbon dioxide, have been made by Calvin et al. (Benson et al., 1950*a*). Identification methods included the use of ion exchange columns and paper chromatography. When tagging occurs during photosynthesis a large number of compounds show activity, even within 30 seconds; after five seconds illumination a large fraction of the activity is confined to four or five compounds. Not all the substances which contain activity and which have been separated by paper chromatography have been identified; a complete analysis of the data is therefore not yet possible. After pre-illumination there is a progressive increase in tagged amino acids for two hours (largely alanine in *Chlorella,* largely aspartic acid in *Scenedesmus*), whereas in photosynthesis the amino acids are conspicuous, even after only five seconds exposure; otherwise compounds labelled after pre-illumination are practically the same as those labelled during brief periods of illumination. After five seconds photosynthesis compounds identified as 2-phosphoglyceric acid and 3-phosphoglyceric acid are predominant amongst labelled compounds. If tagging is continued during a longer period of photosynthesis the relative amount of phosphoglyceric acid rapidly decreases with time; phospho-enol pyruvic acid and later four-

carbon acids begin to appear. After five minutes glucose 1- and 6-phosphates together with fructose 6-phosphate are detectable. Other labelled compounds are sedoheptulose and ribulose phosphates (Benson, 1951). At 4° C tagging during photosynthesis resulted only in the formation of the 2-phosphoglyceric acid compound. Calvin postulated that this was the first identifiable compound formed in photosynthesis.

Evidence that free trioses are the first compounds to be labelled in soybean leaves has been reported by Aronoff (1950). Gaffron (1951) has attributed this to the presence of an active phosphatase in soya bean leaves. The observation that during brief periods of photosynthesis of *Chlorella* in the presence of tracer carbon a large fraction of the activity is initially found in phosphoglyceric acid has been confirmed by the Chicago group (Fager et al., 1950). In the experiments at first reported from Chicago phosphoglyceric acid was not found, and this was attributed to the fact that labelling was not done in the presence of excess carbon dioxide and that consequently the concentration of first intermediates was not maintained during the period of tagging. The Chicago group has now shown that, whereas in the light the ratio of phosphoglyceric to the relatively small amount of pyruvic acid is constant with time, in the dark this ratio declines with time. Since in the light the ratio of phosphoglyceric to phosphopyruvic (5 or 6 to 1) is greater than the equilibrium value (3 to 1), it seems probable that the phosphoglyceric acid is formed prior to phosphopyruvic and hence pyruvic acid. To investigate the fate of phosphoglyceric acid, two samples of cells were pre-illuminated for 15 minutes in nitrogen in absence of tracer, then tracer was added to one in the light and to the other in the dark. Dark fixation resulted in almost exclusive tagging of phosphoglyceric acid (with some pyruvic acid), but light fixation (ten minutes) resulted in a distribution of tracer comparable to that after ten seconds photosynthetic fixation, i.e., the relative amount of phosphoglyceric acid was reduced and the amount of tagged compounds increased. This was taken to be evidence that the phosphoglyceric is photochemically transformed. More correctly it is evidence that phosphoglyceric is transformed as a result of light action, possibly as a result of photosynthesis.

On the basis of his results, assuming that the compounds tagged in the light or in the dark immediately after pre-illumination are related to the photosynthetic process, Calvin has proposed a mecha-

nism of carbon reduction in photosynthesis. He suggests that carbon dioxide combines with a 2C-compound (possibly vinyl phosphate) to give first 2-phosphoglyceric acid and then 3-phosphoglyceric acid which for short periods of illumination is labelled only in the carboxyl group. It is suggested that hexoses are formed from the phosphoglyceric acids. In agreement with this it has been shown that after the first 30 seconds photosynthesis hexoses are labelled at the 3- and 4-C atoms; after a few minutes when the $\alpha$ and $\beta$ atoms of phosphoglyceric are also labelled, the 2- and 5-C atoms and also the 1 and 6 are labelled. This progressive labelling arises, according to Calvin, as the result of the formation of further 2-carbon compound from a cyclic carboxylating process, so that, whereas the first formed hexose derives its activity from the carboxyl group of the phosphoglyceric acid, later this acid is labelled both in the carboxyl and in the 1-C atom and the hexose labelled in all atoms. The cyclic process involves the Wood Werkmann reaction which produces from pyruvic acid, by addition of carbon dioxide, a 4-C atom acid which then splits into two 2-C atom units, one of which may ultimately become hexose, the other continuing the cycle. The details of this cycle are not yet worked out and the evidence for participation of any particular compound is as yet slight. Since tagged succinnate and tagged fumarate are not observed with all the plants examined, which include *Chlorella, Scenedesmus* and barley leaves, it is suggested that the oxaloacetate, formed from pyruvate, may be oxidised to dihydroxymaleic acid which gives rise to glyoxylic and then glycolic acids. Further evidence that a split into 2-C atom units prior to malic acid formation is likely is that addition of malonate, an inhibitor of succinnic dehydrogenase, does not in the main alter the distribution of tagged carbon, except that activity is no longer detected in malic acid (Bassham et al., 1950). From the 4-carbon atom compounds fats and proteins may be synthesized, so that photosynthesis may result in the production of carbohydrates, fats and proteins in a very short time (Clendenning, 1950). That the proportions of these compounds formed during photosynthesis in *Chlorella* can vary within wide limits according to the culture of the cells was known previously (Spoehr and Milner, 1949).

The usefulness of tracer studies in the development of a photosynthetic mechanism depends upon the introduction of a methodology to distinguish those products which are labelled in the

immediate photosynthetic process. As a result of illumination numerous compounds in the plant will change their distribution between different metabolic pathways and may also change in total concentration. Labelled carbon therefore appears in a great variety of compounds. Until detailed kinetic studies are made comparing the rate of the appearance of tracer together with studies of the rate of photosynthesis it will not be possible to distinguish the path of carbon in photosynthesis from the multiplicity of pathways which constitute the metabolism of carbon in the cell.

## SUMMARY

The process of photosynthesis is considered as an oxidation-reduction reaction activated by light absorbed by chlorophyll or certain other substances (e.g., phycocyanin, fucoxanthol), resulting in the evolution of oxygen from water and in the formation of reduced carbon compounds (e.g., carbohydrate) from carbon dioxide. The organisation of the reaction in the cell is not yet understood—the poor photosynthetic activity of light absorbed by chlorophyll in certain red algae may be indicative of a change in this organisation. When chloroplasts are isolated from the cell they are able to catalyse the photochemical reduction of certain hydrogen acceptors with evolution of oxygen from water, but they are unable to reduce carbon dioxide. Analysis of the path of carbon in the green plant cell, both in the light and in the dark, has been made possible by the use of radioactive carbon dioxide. A large number of intermediates in the metabolism of the cell have been identified, but as yet convincing evidence regarding the specific part played by any of these compounds in a particular metabolic process is lacking. The metabolic system of the cell may be analysed in terms of a number of separate processes, but it seems probable that these may have many steps in common. The reactions which must be unique to photosynthesis are those reactions involving at most two light quanta per hydrogen atom transferred which result in the formation of a hydrogen donor capable of reducing carbon dioxide. Comparative studies of the rate of photosynthesis and of the rate of individual steps, as represented for example by the chloroplast reaction and by tracer studies, present the most promising approach to the physiology of photosynthesis.

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# FUNGAL CAROTENOIDS

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Great differences are observed between phanerogams and cryptogams (in particular, fungi) with regard to the occurrence and distribution of carotenoids, and it will serve to place this review of fungal carotenoids in its right perspective if, before considering detailed information, these general differences are outlined.

The carotenoids of green tissues in phanerogams are a complex mixture of carotenes (hydrocarbons) and xanthophylls (oxygen-containing carotenoids), the predominant pigment in each class being β-carotene and lutein (3:3′-dihydroxy-α-carotene), respectively. As far as can be judged, all green tissues contain the same carotenoids, although their quantitative distribution may vary slightly from species to species (see Goodwin (1952*a*) for detailed information). It is only when flowers and fruit are considered that different species begin to display their individuality and to produce carotenoids either species-specific or specific to petals or fruit. Bearing this general picture in mind, a number of striking differences emerge quite clearly when fungal carotenoids are studied:

*a*) Carotenoids are often completely absent from fungi.

*b*) Some species produce only one carotenoid; this is often β-carotene, but this pigment is by no means so universally present in carotenoid-containing fungi as it is in the green tissues of phanerogams.

*c*) Lycopene, which occurs only in fruit and petals but never in green tissues, is often encountered in fungi.

*d*) The xanthophylls characteristic of the phanerogams (often called, somewhat erroneously, phytoxanthins) are rarely detected in fungi; in particular, the most common plant xanthophyll, lutein, has up to the time of writing, never been observed.

*e*) Fungal xanthophylls are usually acidic in character, that is they contain carboxyl groups (e.g., torularhodin) or enolizable hydroxyl groups, as occur in astaxanthin (3:3′-dihydroxy-4:4′-diketo-β-carotene). No acidic carotenoid has ever been detected in phanerogams.

## OCCURRENCE OF CAROTENOIDS IN FUNGI

The investigations to be described in this section on the identification of fungal carotenoids are, considering the size of the problem, woefully few, and if any general conclusions concerning the significance of carotenoid distribution in fungi are to be reached, a great deal more survey work remains to be undertaken. The recent description of a simple spot test for fungal carotenoids (Mrak, Phaff and MacKinney, 1949) may stimulate investigations of this type; care will need to be exercised, however, in applying this test, for carotenoids can be extracted from some fungi, e.g., *Aleuria aurantia*, only with considerable difficulty (Lederer, 1938).

The early dissertations of Zopf (1890) and Kohl (1902) record that unidentified carotenoids are present in the following species which have so far not been recently examined using modern techniques: *Chytridium* spp., *Pilobolus crystallinus, P. kleinii, P. oedipus, Lycogola flavofuscum, Stemonitis* spp., *Pleotrachelus fulgens, Ascobolus* spp., *Leotia lubrica, Nectria cinnabarina, Peziza aurantia, P.* (*Lachnum*) *bicolor, P.* (*Lachnea*) *scutellata, Polystigmum ochraceum* (*fulvum*), *Saccharomyces* spp., *Spathularia flavida, Sphaerostilbe coccaphili, Calocercera cornea, C. palmata, C. viscosa, Coleosporium pulsatilla, Dacromyces stillatus, Ditiola radicata, Melampsora aecidioides, M. salicis caprae, Phragmidium violaceum, Puccinea coronata, Triphragmium ulmariae, Ureo* (*Coleosporium*) *euphrasie* and *Uromyces alchemille.*

Biological assays have shown that vitamin-A active carotenoids are absent from *Alternaria solani, Thielavia terricola, Rhizoctonia solani, Helminthosporium sativum, Fusarium moniliforme, F. oxysporium* and *F. lycopersici* (Gottlieb and Gilligan, 1946). Spectrographic and chromatographic tests have proved that carotenoids are absent from *Polyporus zonalis, P. rubidus, P. grammocephalus, P. luzonensis, Polystictus hirsutus, P. versicolor, P. xanthopus, P. sanguineus, Trametes persooni, T. versatilis, Daedalea flavida, Lenzites subferruginea* and *Ganoderma* (*Formes*) *lucidus* (Bose, 1941), and from *Taphrina deformans, Torulopsis pulcherrima, T. luteola, T. lipofera, Pichia* spp., *Zygosaccharomyces* spp. and *Pullularia* spp. (Mrak et al., 1949). Champeau and Luteraan (1946) have also noted the absence of carotenoids from *Torulopsis pulcherrima.*

Zopf (1890) states that the following fungi produce yellow, orange or red pigments which are not carotenoids (some cited by

Zopf occur in the above list and are not quoted again): *Boletus luridus, B. scaber, Amanita pantherina, A. muscaria, Hygrophorus* (*Hygrocybe*) *conicus, H. punicens, H. coccineus, Cortinarius* (*Inoloma*) *bulliardi, Peziza echinospora, P. sanguinea, Nephoroma lusitanica, Paxillus atrotomentosus, Agaricus* (*Telamoria*) *armillatus, Russula integra, R. emetica, R. alutacea, R. aurata, Gomphidius viscidus, G. glutinosus, Clavaria ternica, Helvella esculenta, Lactarius deliciosus, Thelephorus* spp., *Hydnum ferrugineum, H. repandum, Rhizopogon rubescens, Penicilliopsis clavariaeformis, Phragmidium violaceum* and *Cladonia coccifera.* Zopf (1890) also records the absence of carotenoids from some fungi which produce green (*Peziza aeruginosa*), blue (*Lecidea* spp., *Biatora fungidula, Bilimbia melaena, Bacidia muscorum, Thalloidima candidum*), violet (*Claviceps* spp., *Oidium violaceum, Saccobolus violaceus, Cartinarius violacens, Agaricus laceatus*) and brown (*Lecidea* spp., *Buellia* spp., *Arthonia* spp., *Bachospora dryina, Sarcogyne pruinosa*) pigments. It cannot be assumed, however, that production of a non-carotenoid pigment by a fungus precludes production of a carotenoid, for the two types coexist in *Leotia lubrica, Coleosporium* spp., *Uredo aecidiorides, Nectria cinnabarina* (Zopf, 1890) and *Phycomyces blakesleeanus* (Schopfer, 1943). The more recent definitive work on identification of carotenoids in fungi will now be described.

MYXOMYCETES. *Lycogala epidendron* is the only member of this group which has been examined in detail. Lederer (1938) found that it produces two carotenoids which, although not unequivocally identified, are very probably torulene and rhodoviolascin. Torulene was first encountered in the Rhodotorulaceae (see below), and rhodoviolascin was first isolated from *Rhodovibrio* bacteria by Karrer and Solmssen (1936).

PHYCOMYCETES. Carotene [1] has been detected in the spore-bearing cells of *Pilobolus kleinii* (Bunning, 1937), *Mucor hiemalis* (Chodat and Schopfer, 1927) and *Phycomyces blakesleeanus* (Schopfer and Jung, 1935; Castle, 1935); considerable amounts of carotene also accumulate in the mycelium of *Phycomyces* (Garton,

[1] The term "carotene", used without a prefix, indicates that in the investigation under consideration no separation of constituent carotenes was carried out; normally it connotes a mixture of β-carotene and α-carotene in the ratio of about 9.5 : 0.5.

Goodwin and Lijinsky, 1950, 1951). Schopfer and Jung (1935) considered the pigment in *Phycomyces* to be β-carotene, and, although Castle (1935) reported it as α-carotene, this has been proved quite conclusively by Karrer and Krause-Voith (1947) who showed that α-carotene was present only in traces. These findings have been recently confirmed by Bernhard and Albrecht (1948) and by Garton et al. (1950, 1951). Bernhard and Albrecht (1948) also reported the presence of small amounts of lycopene, and Schopfer and Grob (1950) noted the presence of five unidentified carotenoids in their strain of *Phycomyces*. Using much larger amounts of *Phycomyces*, Goodwin (1951*a*, 1952*b*) has confirmed the presence of lycopene and also identified γ-carotene, ζ-carotene, neurosporene (first obtained by Haxo (1949) from *Neurospora crassa*), phytofluene and phytoene; this is the first time the latter polyene has been observed in fungi. Phytofluene is a colourless polyene containing seven double bonds, five of which are conjugated, and it is characterized by its bright greyish green fluorescence in ultra-violet light and by its three-banded absorption spectrum in the near ultra-violet; it has an empirical formula $C_{40}H_{64\mp2}$ and in all probability has the carotenoid skeleton (Zechmeister and Sandoval, 1946). Porter and Lincoln (1950) think phytofluene is dodecahydrolycopene.

Phytoene was first noted in tomatoes by Porter and Lincoln (1950). It is absorbed on a chromatogram below phytofluene, exhibits a slight blue fluorescence only in very concentrated solutions, and is probably hexadecahydrolycopene.

The relative amounts of the constituent polyenes in *Phycomyces* are: phytoene, 11%; phytofluene, 2%; α-carotene, 0.6%; β-carotene, 84%; γ-carotene, 0.6%; ζ-carotene, 0.6%; neurosporene, 0.6%; lycopene, 0.6% (Goodwin, 1952*b*).

A number of mutant strains of *Phycomyces blakesleeanus* synthesize the same carotenes as does the parent strain (Goodwin and Griffiths, 1952).

Differential accumulations of carotenoids occur in the Mucorales; Lendner (quoted by Satina and Blakeslee, 1926) in 1918 first noted that there was more pigment in the (+) strain of *Mucor hiemalis* than in the (−) strain. This was confirmed by Satina and Blakeslee (1926) and by Chodat and Schopfer (1927); Satina and Blakeslee also showed that this held for a number of other Mucoraceae. Schopfer (1943) suggested that the same situation held for

the (+) and (−) types of *Phycomyces blakesleeanus,* but Garton et al. (1950, 1951) found that their (−) strain always produced about twice as much carotene as did their (+) strain, irrespective of variations in cultural conditions.

Emerson and Fox (1940) examined the carotenoids of some species of the aquatic phycomycete *Allomyces* with interesting and important results. According to these authors, *Allomyces* spp. can be divided into two types, *Euallomyces,* which show marked morphological alternation of generations, and *Cystogenes,* which produce cysts but go through no obvious sexual phase. The cystogene *A. cystogena* produces no carotenoids, but *A. moniliformis* (also a cystogene) produces γ-carotene and distributes it widely and indiscriminately in sporangia, hyphae and spores. The asexual plants and the female forms of the Euallomyces, *A. arbuscula, A. javanicus* and *A. macrogyna,* contain no carotenoids, whilst the male forms produce γ-carotene and accumulate it specifically in gametangia, the pigment persisting in the gametes after emergence from the gametangia. Traces of another pigment always occurred with γ-carotene, and in *A. javanicus* this was identified as β-carotene. No xanthophylls were ever detected.

ASCOMYCETES. Lederer (1938) has confirmed the early report of Zopf (1890) that *Polystigma rubrum* contains two carotenoids; one is in all probability lycoxanthin (3:hydroxylycopene), first isolated from tomatoes by Zechmeister and Cholnoky (1936), whilst the other is an unidentified acidic carotenoid exhibiting an ill-defined absorption spectrum with maxima (in $CS_2$) at 550 and 515 mμ. Kohl's (1902) early spectroscopic data on *Nectria cinnabarina* suggest that this may contain similar pigments.

Haxo (1949) found that *Neurospora crassa* produces a complex mixture of carotenoids, most of which are epiphasic.[2] Both the wild type and a non-conidiating mutant #580 produce the same pigments. The four major epiphasic pigments were obtained crystalline and identified as lycopene, γ-carotene, rhodoviolascin and neurosporene. This last pigment is a new carotenoid, $C_{40}H_{58\mp2}$, m.p. 123.8°, which contains nine conjugated double bonds. Porter

[2] When a light petroleum solution of a mixture of carotenoids is shaken with 90% aqueous methanol, the carotenoids are partitioned between the two solvents. The hydrocarbons, monohydroxy and monoketo derivatives remain in the petrol layer (the epi-phase), whilst the more highly oxygenated derivatives are transferred to the methanol (the hypophase).

and Lincoln (1950) have recently reported that in the near future they will present evidence to show that neurosporene is identical with tetrahydrolycopene which occurs in tomatoes. Haxo actually identified his third carotenoid as spirilloxanthin, a pigment first isolated from *Rhodospirillum rubrum* by van Niel and Smith (1935). Recently, however, Polgar, van Niel and Zechmeister (1944) have concluded that there is "good reason for admitting" that spirilloxanthin is identical with rhodoviolascin (Karrer and Solmssen, 1936). Of the four minor epiphasic components, only β-carotene was unequivocally identified, the remaining three closely resembled lycoxanthin (or perhaps rhodopin), α-carotene and rhodopurpurene. Rhodopin and rhodopurpurene are two further pigments occurring in the purple bacteria; their structures are not certain (Karrer and Solmssen, 1936). The major constituent of the hypophasic pigments of *Neurospora crassa* is an unidentified acidic carotenoid. Phytofluene was also detected. Heim (1946) reported the presence of (unidentified) carotenoids in the following Discomycetes: *Sarcoscypha coccinea, Peziza aurantia, Melastiza miniata, Anthracobia melaloma* and *Humaria* spp.

BASIDIOMYCETES. As early as 1886 Müller (quoted by Lederer, 1938) recorded the presence of crystalline lipochromes in uredospores. This was confirmed a little later (1892) by Bertrand and Poirault, but no further progress was reported until Lederer (1938) examined four species in detail. *Puccinia coronifera* produces α-, β- and γ-carotenes and an acidic pigment which was not identified completely but which appeared to be rhodotorulin (see later). Lederer (1938) also detected α-, β- and γ-carotenes in *Coleosporium senecioni*, and *Tremella mesenterica*, on the other hand, produces only β-carotene, whilst from the related *Aleuria aurantia* a mixture of pigments is obtained consisting of α- and β-carotene, a pigment provisionally identified as rubixanthin, and an unidentified pigment. Rubixanthin (3-hydroxy-γ-carotene) was first isolated from rose hips by Kuhn and Grundmann in 1934.

Heim (1946) has demonstrated the presence of carotenoid crystals in the cytoplasm of *Mutinus caninus, M. bambusinus, Lysurus hexagonus* and of *Pseudocolus* obtained from Madagascar. In *M. caninus*, oily droplets also occur, but Heim considers that this is more characteristic of the Ascomycetes.

Willstaedt (1937) obtained some interesting results from a study of the carotenoids of three species of *Cantharellus*. *C. cibarius* pro-

duces mainly β-carotene (4 mg.% fresh wt.), but small amounts of α-, γ- and δ-carotenes and lycopene were also encountered. *C. lutescens* and *C. infundibiliformis,* in contrast, produce considerable amounts of lycopene but no β-carotene. *C. infundibiliformis* contains a small amount of a pigment which Willstaedt has tentatively identified as β-apo-8′-carotenal. δ-Carotene, of unknown constitution, was first noticed in the seed hulls of *Gonocaryum pyriforme* (Winterstein, 1933) and β-apo-8′-carotenal is one of a group of compounds which Karrer and his school (see Karrer and Jucker, 1948) have prepared by controlled oxidation of naturally-occurring carotenoids. Except in the investigation under consideration, apo carotenoids have never been detected in Nature. It is, therefore, important for this observation to be either confirmed or refuted, for what appeared to be an apo-carotenoid in 1937 might now be revealed, using modern knowledge and techniques, as a carotenoid epoxide or more probably neurosporene.

Haxo (1951) has recently examined the related *Cantharellus cinnabarinus.* The presence of β-carotene and phytofluene was established and a new pigment canthaxanthin was described. Two other new pigments were observed adsorbed just above β-carotene and lycopene respectively, but they were not obtained in amounts sufficient for crystallization.

Goodwin (1951*b*) has found the following polyenes in *Dacromyces stillatus,* phytofluene, α-, β-, γ- and ζ-carotenes, torulene, cryptoxanthin and zeaxanthin.

The telial galls of the rust fungus *Gymnosporangium juniperi-virginianae* contain γ- and β-carotene, the former predominating. Traces of xanthophylls are also present (Smits and Peterson, 1942).

SCHIZOMYCETES. Of the two families of Anascosporogeneous yeasts, the Rhodotorulaceae contain carotenoids, whilst the Torulopsidaceae do not. Zopf (1890) first noted the presence of lipochromes in the red yeasts, and in 1916 Chapman reported that the spectrum of the pigment was different from that of carotene. Nothing further was reported until Lederer (1933, 1938) carried out his important work on *Rhodotorula rubra.* Specimens of *R. rubra* from three different sources contained the same four pigments, viz.: an acidic pigment, β-carotene, torulene and a pigment which was very unstable and could not be examined in detail.

Lederer (1933, 1938) suggests that the torulene is 3:3′-dimethoxy-γ-carotene but emphasizes that conclusive proof is lacking. This structure would fit in with the observation that torulene is inactive as a vitamin-A precursor (Joué and Chaix, 1938). Karrer and Rutschmann (1946) reinvestigated the acidic fraction and concluded that it is a carboxy carotenoid. They named it "torularhodin".

Although torularhodin was the major pigment in Karrer and Rutschmann's cultures, Fink and Zenger (1934) could detect it only in small amounts in their cultures of *R. rubra,* and this was borne out by the work of Bonner, Sandoval, Tang and Zechmeister (1946) who could find practically none in their cultures. Bonner et al. (1946) found, in addition to β-carotene and torulene, small amounts of two other pigments; one was identified as γ-carotene and the other, first thought to resemble β-carotene-5:6,5′:6′-diepoxide, as neurosporene (Haxo, 1949). A pigment similar to auroxanthin (5:8,5′:8′-diepoxyzeaxanthin) but now known to be ζ-carotene was noted in one mutant strain but apparently not in the original strain. Bonner et al. also noted the presence of phytofluene in *R. rubra.* The concentrations of torulene, neurosporene, α-carotene, β-carotene and phytofluene in their original strain were, respectively, in mg.% dry yeast: 5.2, 0.28, 0.65, 0.74 and 0.69.

Fromageot and Tschang (1938), examining the related *Rhodotorula sanniei,* found torularhodin to be the predominant pigment, the amounts of torularhodin, torulene and β-carotene obtained from 100 g. of dried yeast being 290, 14.3 and 1 mg., respectively; γ-carotene and lycopene were present only in traces. *Rhodotorula gracilis* is reported to have a vitamin-A activity of 5–10 i.u./g. (dry wt.) (Nillson, Enebo and Brunius, 1942). This is consistent with a β-carotene and torulene content of the same order as that noted in other Rhodotorulaceae.

*Sporobolomyces roseus* and *S. salmonicolor* both contain torulene and torularhodin but no β-carotene; the pigments are extracted only with difficulty, and then not completely (Lederer, 1938).

The foregoing account is summarized in the following table.

## BIOGENESIS OF CAROTENOIDS

GENERAL FACTORS. The possibility has long been entertained that the phytyl residue of the chlorophyll molecule might be the

source of carotenoids in green plants, but recent work, especially on excised fruit (see Goodwin, 1952*a*), has made this extremely improbable. In fungi, however, this possibility does not exist, and this fact, combined with others such as the absence of photosynthetic processes and the ease with which some fungi will grow on completely defined media to produce single or simple mixtures of carotenoids, makes them ideal organisms for studying carotenoid biogenesis. Information concerning the detailed mechanism of synthesis is still almost non-existent, but progress is being slowly made. On the other hand, a considerable amount of data is available concerning the general nutritional and cultural factors controlling carotenoid production.

The general pattern of pigment production noted in *Rhodotorula rubra* by Luteraan and Choay (1947) can be divided into three phases: a period of active synthesis leading to maximum production, a period of persistence during which no change in content occurs, and finally a period during which the pigments gradually disappear. The onset of the third phase can be delayed indefinitely by adding oleic acid to the medium and can be accelerated by addition of ammonium sulphoricinoleate. Garton et al. (1950, 1951) have noted the same general pattern in *Phycomyces* but found that the phase of active synthesis can be further sub-divided into two sub-phases. In the first sub-phase (i.e., the first four or five days of growth), $\beta$-carotene synthesis is quite slow compared with growth (dry wt. production) and lipid synthesis; between five and ten days (second sub-phase), however, the rate of $\beta$-carotene synthesis is very much greater. The onset of this period of rapid synthesis coincides with cessation of growth as measured by either dry weight production or nitrogen assimilation (Goodwin, Lijinsky and Willmer, 1951; Goodwin and Willmer, 1952).

CARBON SOURCES. Different carbohydrates vary in their ability to promote carotenogenesis. In *Rhodotorula sanniei,* for example, glycerol is the most effective single source, although a mixture of lactic acid and glucose is equally effective. Glucose alone does not support pigmentation, for, using gelatin as the nitrogen source, it will initiate but not maintain pigmentation, and eventually a colourless *R. sanniei* is produced. This form has not, however, lost its ability to synthesize carotenoids, for when transferred to an ade-

TABLE I

DISTRIBUTION OF CAROTENOIDS IN FUNGI

| Pigment | Source | Reference |
|---|---|---|
| α-Carotene | *Aleuria aurantia* | Lederer (1938) |
| | *Cantharellus cibarius* | Willstaedt (1937) |
| | *Coleosporium senecionis* | Lederer (1938) |
| | *Dacromyces stillatus* | Goodwin (1951*b*) |
| | *Neurospora crassa* | Haxo (1939) |
| | *Phycomyces blakesleeanus* | Karrer and Krause-Voith (1947) |
| | | Bernhard and Albrecht (1948) |
| | | Garton et al. (1950) |
| | *Puccinia coronifera* | Lederer (1938) |
| | *Rhodotorula rubra* | Lederer (1938) |
| | | Fromagoet and Tschang (1938) |
| | | Bonner et al. (1946) |
| β-Carotene | *Aleuria aurantica* | Lederer (1938) |
| | *Allomyces* (in traces) | Emerson and Fox (1940) |
| | *Cantharellus cibarius* | Willstaedt (1937) |
| | *Cantharellus cinnabarinus* | Haxo (1950) |
| | *Dacromyces stillatus* | Goodwin (1951*b*) |
| | *Gymnosporangium juniperi-virginianae* | Smits and Peterson (1942) |
| | *Neurospora crassa* | Haxo (1949) |
| | *Phycomyces blakesleeanus* | Karrer and Krause-Voith (1947) |
| | | Bernhard and Albrecht (1948) |
| | | Garton et al. (1950) |
| γ-Carotene | *Allomyces* spp. | Emerson and Fox (1940) |
| | *Cantharellus cibarius* | Willstaedt (1937) |

TABLE I (*continued*)

| Pigment | Source | Reference |
|---|---|---|
| γ-Carotene (*continued*) | *Coleosporium senecionis* | Lederer (1938) |
| | *Dacromyces stillatus* | Goodwin (1951*b*) |
| | *Gymnosporangium juniperi-virginianae* | Smits and Peterson (1942) |
| | *Neurospora crassa* | Haxo (1949) |
| | *Phycomyces blakesleeanus* | Bernhard and Albrecht (1948) |
| | | Goodwin (1951*a*, 1952*b*) |
| | *Puccinia coronifera* | Lederer (1938) |
| | *Rhodotorula rubra* | Lederer (1938) |
| | | Fromageot and Tschang (1938) |
| | | Bonner et al. (1946) |
| | *Rhodotorula sanniei* | Fink and Zenger (1938) |
| | *Tremella mesenterica* | Lederer (1938) |
| β-apo-8′-carotenal | *Cantharellus infundibiliformis** | Willstaedt (1937) |
| β-carotene-5:6,5′:6′-diepoxide | *Rhodotorula rubra** | Bonner et al. (1946) |
| ζ-Carotene | *Phycomyces blakesleeanus* | Goodwin (1952*a*) |
| | *Phycomyces nitens* | Goodwin and Griffiths (1952) |
| | *Dacromyces stillatus* | Goodwin (1951*b*) |
| δ-Carotene | *Cantharellus cibarius* | Willstaedt (1937) |
| Lycopene | *Cantharellus cibarius* | |
| | *Cantharellus infundibiliformis* | Willstaedt (1937) |
| | *Cantharellus lutescens* | |

*Indicates that the pigment was not unequivocally identified.

TABLE I (*continued*)

| Pigment | Source | Reference |
|---|---|---|
| Lycopene (*continued*) | *Neurospora crassa* | Haxo (1949) |
| | *Phycomyces blakesleeanus* | Bernhard and Albrecht (1948) |
| | | Goodwin (1950*a*) |
| Lycoxanthin | *Neurospora crassa** | Haxo (1949) |
| | *Polystigma rubrum** | Lederer (1938) |
| Neurosporene | *Neurospora crassa* | Haxo (1949) |
| | *Rhodotorula rubra* (pigment A) | Bonner et al. (1946) |
| | | Haxo (1949) |
| | *Phycomyces blakesleeanus* | Goodwin (1950*a*) |
| Phytofluene | *Cantharellus cinnabarinus* | Haxo (1949) |
| | *Dacromyces stillatus* | Goodwin (1951*b*) |
| | *Neurospora crassa* | Haxo (1949) |
| | *Phycomyces blakesleeanus* | Goodwin (1950*a*) |
| | *Rhodotorula rubra* | Bonner et al. (1946) |
| Rhodopin | *Neurospora crassa** | Haxo (1949) |
| Rhodotorulin | *Puccinia coronifera** | Lederer (1938) |
| | *Rhodotorula rubra* | Lederer (1938) |
| | | Fromageot and Tschang (1938) |
| | | Karrer and Rutschmann (1943, 1946) |
| | | Bonner et al. (1946) |
| | *Rhodotorula sannei* | Fink and Zenger (1938) |
| | *Sporobolomyces roseus* | Lederer (1938) |
| | *Sporobolomyces salmonicolor* | Lederer (1938) |
| Rhodopurpurin | *Neurospora crassa* | Haxo (1949) |

TABLE I (*continued*)

| Pigment | Source | Reference |
|---|---|---|
| Rhodoviolascin | *Lycogola epidendron** | Lederer (1938) |
| | *Neurospora crassa* | Haxo (1949) |
| Rubixanthin | *Coleosporium senecionis** | Lederer (1938) |
| Torulene | *Dacromyces stillatus* | Goodwin (1951*b*) |
| | *Lycogola epidendron** | Lederer (1938) |
| | *Rhodotorula rubra* | Lederer (1938) |
| | | Fromageot and Tschang (1938) |
| | | Karrer and Rutschmann (1943) |
| | | Bonner et al. (1946) |
| | *Rhodotorula sanniei* | Fink and Zenger (1938) |
| | *Sporobolomyces roseus* | Lederer (1938) |
| | *Sporobolomyces salmonicolor* | Lederer (1938) |
| Canthaxanthin | *Cantharellus cinnabarinus* | Haxo (1950) |

quate medium it assumes its original colour (Fromageot and Tschang, 1938).

Maltose and glucose were equally effective as carbon substrates for carotenogenesis in *Phycomyces blakesleeanus,* but xylose and fructose were considerably less effective, although equally good in promoting general growth and lipid synthesis. Lactose and glycerol were ineffective merely because the fungus will not grow on media containing either of these as the sole carbon source (Garton et al., 1951). Incidentally, in contrast to these observations, Schopfer (1934) found that his *Phycomyces* grew reasonably well in glycerol.

Schopfer and Grob (1950) found that *Phycomyces* would grow, although only slightly, and produce no carotene on a medium containing ammonium lactate as the sole carbon and nitrogen source; addition of acetate to this medium stimulated growth to a certain extent but stimulated carotenogenesis to a much greater degree; this was comparable to that produced on a medium containing glucose, which, however, stimulates growth to a much greater extent. The same effect was observed with *Rhodotorula rubra* and *Mucor haemalis* (Grob, Poretti, von Muralt and Schopfer, 1951). Palmitic acid, citric acid and tartaric acid are also active, whilst glycerol, acetone and $\beta$-methylcrotonic acid ester are inactive.

Recently Goodwin and Willmer (1951) have repeated the experiments of Schopfer and Grob (1950) with acetate and have broadly confirmed their observations. If, however, well-formed mycelia are transferred to the media described by Schopfer and Grob (1950), the stimulatory effect disappears (Glover, Goodwin and Lijinsky, 1951).

If well-formed mats of *Phycomyces* are transferred to a medium containing glucose but no nitrogen, the carotene synthesis is relatively much greater than if nitrogen is present (Goodwin, Lijinsky and Willmer, 1951; Goodwin and Willmer, 1952). The original suggestion (Garton et al., 1951) that the fungus had to be dissimilating nitrogen before carotene could be produced, was based on a mis-interpretation of the experimental results (see section on pH).

NITROGEN SOURCES. Schopfer (1935) reported that glycine and asparagine were equally effective in promoting carotene synthesis in *Phycomyces* but that ammonium nitrate was better than either. Garton et al. (1950, 1951) examined these compounds in more

detail and also tested L-valine, L-leucine, L-isoleucine and L-alanine as well as ammonium acetate. In media containing 3% glucose carotenogenesis was essentially the same on all these nitrogen sources except glycine; this amino-acid specifically stimulated carotene synthesis, producing mycelia containing 200 mg.% (dry wt.) of β-carotene compared with the usual 120–140 mg.%. More recent experiments (Goodwin and Lijinsky, 1951) have shown that using sub-optimal concentrations of glucose (1%), L-valine and L-leucine strongly enhance carotene production, whilst under these conditions, glycine and other amino acids are ineffective.

Méry (1949) records qualitative experiments which suggest that tyrosine stimulates considerably both lipogenesis and carotenogenesis in the red yeasts.

SIGNIFICANCE OF THE C : N RATIO IN CAROTENOGENESIS. Schopfer's (1935, 1943) pioneer experiments on carotene production by *Phycomyces blakesleeanus* and *Mucor hiemalis* suggested that the C : N ratio of the medium is an important factor, if not the governing factor in carotenogenesis. Similar suggestions exist to explain lipogenesis in microorganisms (see Kleinzeller, 1948, for a review). Garton et al.'s (1951) investigations on *Phycomyces* show that the C : N ratio can often assume a spurious significance. Provided sufficient nitrogen is available to allow maximum mycelial growth, the controlling factor is not the C : N ratio but the amount of assimilable carbon available after growth (nitrogen assimilation) has stopped. For example, maximal carotene production on a medium containing 1.5% glucose and sufficient nitrogen for maximal growth (0.2% asparagine) is 20–30 mg.% dry weight; when the glucose content is increased to 2.5% and the asparagine content is maintained at 0.2%, the carotene production increases to 120–150 mg.%. On the other hand, if the glucose content is maintained at 2.5%, variation of asparagine concentration from 0.1 to 1.0% has no effect on carotene production.

Goodwin, Lijinsky and Willmer (1951) have shown that during active growth (i.e., during nitrogen assimilation) of a *Phycomyces* culture, β-carotene is synthesized only very slowly and that it is not until growth (N assimilation) is complete (under the conditions of this experiment, three to four days) that production is accelerated. The probable explanation is that during growth most of the glucose

assimilated goes in the synthesis of structural material, but that when growth is complete the fungus dissimilates glucose with production by fermentative assimilation of, amongst other compounds, carotene.

EFFECT OF LIGHT. Deventer (quoted by Zechmeister, 1944) found that light was necessary for carotenoid synthesis by *Neurospora sitophila,* and Schopfer and Jung (1935) stated that blue light is necessary for carotene synthesis by *Phycomyces,* none being produced in the dark or in red light. Haxo (1949), on the other hand, found that light was not essential for carotenoid production by *Neurospora crassa,* but that it did stimulate synthesis considerably, the spectral region 520–580 mμ being most effective. Garton et al. (1950, 1951), using *Phycomyces,* have not been able to reproduce Schopfer and Jung's observations. Numerous experiments have indicated that *Phycomyces* cultured in the dark always produces about one-half the amount produced under similar conditions in light; furthermore, the wavelength of the illumination does not appear to be of importance. Although stimulating carotenoid production in *N. crassa,* light did not stimulate the synthesis of phytofluene (Haxo, 1949). In *Phycomyces,* on the other hand, recent experiments have indicated that light exerts the same effect on phytofluene synthesis as on carotene synthesis (Goodwin, 1951*a*, 1952*a*).

EFFECT OF TEMPERATURE, $O_2$ AND pH. Fromageot and Tchang (1938) found that pigmentation in *Rhodotorula sanniei* remains qualitatively the same within the temperature limits 14–28° and the pH limits 5.2–7.6. Luteraan and Dieng (1948) noted that pigment (? carotenoid) production in *Saccharomyces cerevisiae* depends upon the $O_2$ tension of the environment, and Méry (1948) noted a similar effect with *Rhodotorula gracilis.*

Goodwin, Lijinsky and Willmer (1951) and Goodwin and Willmer (1952) have noticed that as *Phycomyces* develops, the pH of the medium drops from about 6 to 3.2. If this fall is prevented by buffering the medium at a pH of 7.0, 6.0 or 5.2, carotenogenesis is considerably inhibited, although growth of the fungus is not affected. This inhibition is also observed if mats are transferred to buffered media.

TRACER EXPERIMENTS. Grob et al. (1951), using $C^{14}$, have shown that the carbon of both the methyl and the carboxyl group

of acetate is incorporated into β-carotene by *Phycomyces.* The carboxyl carbon was found to be twice as effective as the methyl carbon. Glover, Goodwin and Lijinsky (1951) and Glover, Goodwin and Willmer (1951) have confirmed that the methyl carbon of acetate is incorporated into the carotene molecule, but preliminary experiments (Glover, Goodwin and Willmer, 1951) have not yet demonstrated any incorporation of labelled $CO_2$ into the carotene molecule.

INHIBITION STUDIES. Kharasch, Conway and Bloom (1936) noticed that chromogenesis in many bacteria and fungi was inhibited by the presence of diphenylamine, and Turian (1950) has shown that in *Mycobact. phlei* carotenogenesis is inhibited by 1/35,000 diphenylamine. Goodwin (1951*b*, 1952) found that in *Phycomyces,* whilst synthesis of β-carotene is considerably inhibited by diphenylamine, that of the more saturated members of the series, phytofluene, ζ-carotene and neurosporene is considerably stimulated.

Goodwin and Lijinsky (1951*b*) have investigated a large number of potential inhibitors, but none has been found either to stimulate or inhibit carotenogenesis in *Phycomyces;* included in their study was phenol and 2:4-dinitrophenol which Turian (1951) found, respectively, to inhibit and to stimulate carotenogenesis in *Mycobact. phlei.*

The mode of action of diphenylamine is not yet known, but Goodwin and Willmer (1951) have found that adenosine monophosphate, when added to a medium containing diphenylamine, returns the β-carotene synthesis to normal but does not reduce the amount of phytofluene, ζ-carotene and neurosporene accordingly. Streptomycin reduces the synthesis of all carotenes equally in *Phycomyces,* the levels being about 30% of normal. No effect is observed on growth and lipogenesis (Goodwin and Griffiths, 1952).

## MISCELLANEOUS EXPERIMENTS

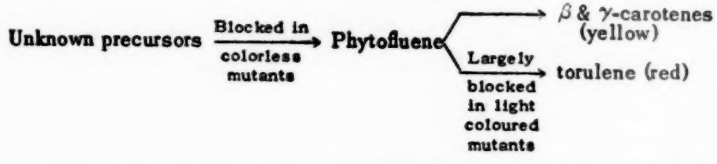
According to Luteraan and Dieng (1948), exposure of *Rhodotorula* spp. to the vapours of camphor, terpineol or menthol, results in decolorization of the fungus within 24 to 48 hours. If these cultures are then washed free from the terpenes and transferred to a new medium, rapid and abnormal growth and pigmentation occur. *Saccharomyces cerevisiae,* which normally does not produce carotenoids, takes on a red tinge when treated in the same way.

Luteraan and Dieng (1948) assume that this colour is due to a carotenoid, but no supporting evidence is supplied. Steroids have no such action on either *Rhodotorula* or *Saccharomyces* but oxygenation produces a similar reaction in *Saccharomyces*.

Luteraan and Choay (1947) report that an unspecified species of *Penicillium* grown on glycerol in the rigorous absence of aneurin produces a large amount of carotenoid instead of the usual non-carotenoid pigment. No experimental details are given, and confirmation of this striking observation is awaited with interest.

EXPERIMENTS WITH MUTANT STRAINS. Rather surprisingly, only one investigation on carotenogenesis using mutants has been reported. Bonner et al. (1946) examined seven ultra-violet mutants of *Rhodotorula rubra*. In three red mutants the amounts of all constituent carotenoids except torulene were approximately doubled, that of torulene increased only slightly. In a brownish orange (II) and a pale orange (I) mutant torulene had almost entirely disappeared. Differences between these two mutants were, however, observed; in I the neurosporene level was normal and in III it was higher than in the red mutants IV, V and VI; $\beta$-carotene was normal in I and at the "red" level in II; $\gamma$-carotene was normal in I and slightly enhanced in II; phytofluene was normal in II and enhanced in I; $\zeta$-carotene (?) appeared in II but not in I. The two colourless mutants produced no carotenoids.

From these results Bonner et al. have suggested the following working hypothesis for carotenogenesis in *Rhodotorula:*

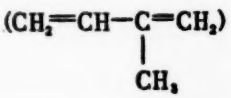



It should be emphasized that, although this scheme does not clash with the reported facts, many other possibilities, as Bonner et al. admit, exist. Chief amongst these is the possibility that phytofluene is not a precursor of the carotenoids; it may be produced colaterally with the pigments or may even be a degradation product. In this connection it is interesting to find that after two and one-half years of monthly transfers on agar, the phytofluene content of

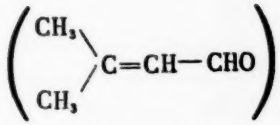
these mutants has markedly diminished, whilst that of the other carotenoids has remained unchanged (Tang, Bonner and Zechmeister, 1949).

### CAROTENOID PRECURSORS

Information concerning carotenoid precursors in both higher plants and fungi is meagre, but speculation concerning the mode of synthesis has not been lacking. When, as stated at the beginning of this section, the phytyl residue from chlorophyll was shown not to be the immediate source of carotenoids in the higher plants, first isoprene

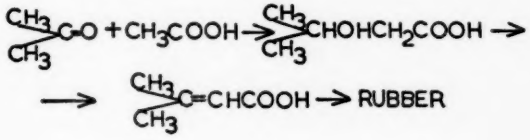


(Kuhn, Winterstein and Wiegand, 1928) and then β-methylcrotonaldehyde



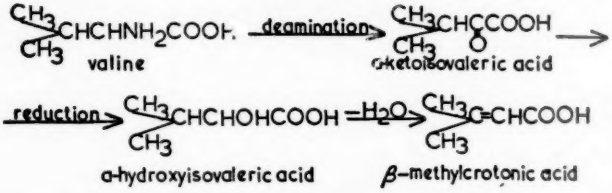
(Karrer, Helfenstein and Widmer, 1928) were suggested as the fundamental repeating unit; neither of these compounds occurs naturally.

No confirmation or otherwise of these hypotheses has yet been forthcoming, but recently Bonner and his associates (see, for example, Bonner and Arreguin, 1949) have investigated the synthesis of rubber, which is an isoprenoid polymer. In culture experiments they found that rubber synthesis was greatest when a mixture of acetone and acetate was used as the carbon source. These two compounds can theoretically condense to form β-methylcrotonic acid, and it was found that this compound was in fact active, but not to the same degree as a mixture of acetone and acetate:

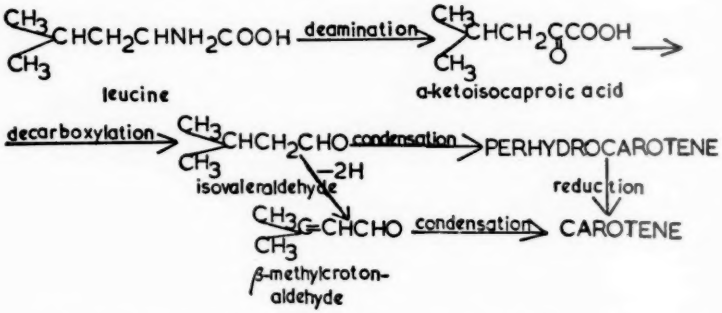


In this connection it is important to note that β-methylcrotonaldehyde is inactive.

The possibility that this type of reaction is concerned in the synthesis of carotenoids is strengthened by the recent experiments of Goodwin and Lijinsky (1951*a*). Culturing *Phycomyces* on a sub-optimal amount of carbon (1% glucose), they have found that of the amino-acids so far examined, only L-valine and L-leucine stimulate the synthesis of β-carotene. Glycine, which stimulates carotenogenesis when the glucose concentration is optimal (3%), does not function at the lower glucose concentration. Valine could be metabolized in the following way to yield β-methylcrotonic acid:




Leucine, which is much more active than valine, presents an interesting problem, and it appears that what most likely happens is the following, *Phycomyces* having the ability to decarboxylate β-keto *iso*-caproic acid to isovaleraldehyde in the same way as do yeasts in the production of fusel oil:




With reference to this scheme, Glover, Goodwin and Lijinsky (1951) have recently found that α-ketoisocaproic acid and β-methylcrotonaldehyde stimulated carotenogenesis, whilst isovaleraldehyde was much less active; β-methylcrotonic acid was inactive. Prelimi-

nary work using acetate labelled with $C^{14}$ in the methyl group suggests that isovaleraldehyde is not an intermediate in the incorporation of acetate into carotene in *Phycomyces* (Glover, Goodwin and Willmer, 1951). This of course does not rule out the fact that it may be an intermediate in the series of reactions leucine → carotene. All these suggestions are based on the supposition that β-methylcrotonaldehyde functions as the repeating unit in carotenoid synthesis. This is, of course, far from proved, but the preliminary work reported here does suggest that it or some closely related compound is the key intermediate in this synthesis. The possibility that a saturated compound, such as isovaleraldehyde or α-hyroxyisovaleric acid, is the intermediate, should not be overlooked; this would probably entail the production of a saturated $C_{40}$ compound which would then be desaturated with the formation of a carotenoid. Porter and Lincoln (1950) have built up a very elaborate theory of carotenogenesis in tomato fruit on the assumption that carotenoids are formed by the stepwise removal of four hydrogen atoms from a saturated parent compound.

The work of Goodwin (1951*a*, 1952*b*) on the effect of diphenylamine on carotenogenesis at first sight appears to support the hypothesis of Porter and Lincoln (1950), but it should be pointed out that other explanations are possible (Goodwin, 1952*b*). The observation that isovaleraldehyde is probably not an intermediate in the series of reactions acetate → carotene (Glover, Goodwin and Willmer, 1951) should also be taken into account, but obviously much more work is required before this problem is finally solved. It should be recalled that Hilditch (1951) considers that the evidence available concerning the synthesis of unsaturated fatty acids points away from their being formed from saturated precursors.

The role of glycine in carotenogenesis in the presence of optimal amounts of sugar (Garton et al., 1950) remains unexplained; but it may be connected with the recent observations that it is an active source of labile methyl groups (Arnstein and Neuberger, 1950).

It will be obvious from this discussion that up to the present only the surface of the problem of carotenogenesis in fungi or other organisms has been scratched. Many problems remain, for example, do four $C_5$ (? β-methylcrotonaldehyde) units combine head to tail to form a $C_{20}$ unit, two of which then combine tail to tail to form a carotenoid, or is the sequence of events a condensation, tail

to tail, followed by head to tail condensations at either end of this unit? These can be solved only by a great deal more work, and it seems to the reviewer that in this connection fungi represent ideal experimental material.

## FUNCTION OF CAROTENOIDS IN FUNGI

It has long been considered that carotenoids function in fungi in phototropic responses (see Wald, 1943, for a review). Blaauw in 1909 (quoted by Wald) obtained an action spectrum for the phototropic bending of *Phycomyces* with a maximum of 495 mμ. Later repetition of this work by Castle (1935), Bünning (1937) and Buder (1932—quoted by Wald) showed that the maximum of the band, which was broad, actually occurred in the spectral region 440 mμ. Similar results were recorded by Bünning (1937) for *Pilobolus kleinii.* It has also been shown by the above mentioned workers that β-carotene exists in the light-sensitive zones of these fungi.

Recently Galston (1950) has provided convincing evidence that in the higher plants riboflavin could with equal justification be considered as the photo-active pigment. Briefly the argument is based on the fact that measurements of action spectra are not sufficiently precise to distinguish between riboflavin and carotenoids, that riboflavin as well as β-carotene exists in the light-sensitive zones and that riboflavin can affect growth by sensitizing the photodestruction of indole acetic acid (Galston, 1949) in the same way as β-carotene catalyzes the photodecomposition of auxin-a-lactone (Kögl and Schuringa, 1944).

Whether this argument can be extended to include fungi awaits further evidence. A pointer in this direction is that 96% of the auxin activity of *Phycomyces* sporangiophores is due to indole acetic acid (Kögl and Verkaark, 1944). On the other hand, auxin (indole acetic acid or auxin-a) it not necessary for the growth of, for example, *Phycomyces,* although indole acetic acid does stimulate early growth of this fungus by about 10% (Richards, 1949), and, further, the presence of riboflavin has not yet been demonstrated in the photosensitive regions of fungi. *Phycomyces* grown in the presence of diphenylamine and containing about 3% of its normal amount of β-carotene is still strongly phototropic (Goodwin and Lijinsky, 1951*b*).

A great deal of circumstantial evidence is available suggesting that carotenoids function in reproduction in many plants and animals; the direct evidence is, however, much less (see Goodwin, 1950*b*, for a review). When fungi are considered from this point of view, the possibility of carotenoid mediation in reproduction is derived solely from investigations which show that different sexual forms either produce specific carotenoids and accumulate them in the gametangia or accumulate different amounts of the same carotenoids. Emerson and Fox (1940), as stated previously, have shown that only the male forms of the Euallomyces contain carotenoids ($\gamma$-carotene) and that these are confined to the gametangia. The non-sexual cystogenes generally produce no carotenoids, but when they do there is no specific accumulation in one organ, they are indiscriminately scattered throughout the fungus. The (+) and (−) strains of the Mucoraceae produce different amounts of the same carotenoid, the (+) always more. Similar observations were recorded for *Phycomyces blakesleeanus* by Schopfer (1934) and Garton et al. (1951), although a difficulty arises in this case because Schopfer's (+) strain produced the more pigment, whilst Garton et al.'s (−) strain had the higher concentration. Much further work is required before it can be definitely assumed that the widespread differential accumulation of carotenoids in different sexes (strains) of the same species is of fundamental importance in reproduction in fungi.

In the Rhodotorulaceae carotenoids are apparently dissolved in the pericapsular fat and do not occur in the fatty globules (Champeau and Luteraan, 1946). This also suggests a function, and Champeau and Luteraan have considered that this may be respiratory.

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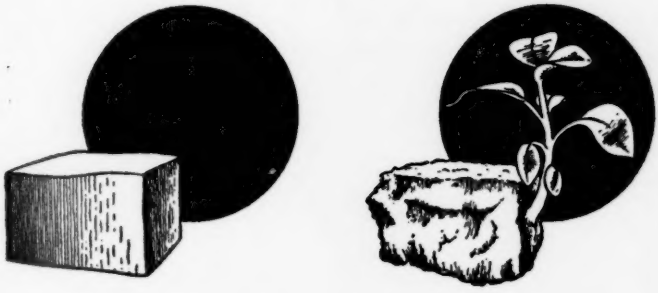


Fig. 19-6. The application of adenine to excised tobacco stem tissue or stem callus results in the initiation of adventitious buds.

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